WEST Search History

DATE: Tuesday, August 19, 2003

Set Name side by side	Query	Hit Count	Set Name result set
	DB=USPT,PGPB,JPAB,EPAB,DWPI,TDBD; PLUR=YES; OP=AND		
L1	edb or ed-b	505	L1
L2	L1 and fibronectin	73	L2
L3	('6093399')[ABPN1,NRPN,PN,TBAN,WKU]	2	L3
L4	('6051230')[ABPN1,NRPN,PN,TBAN,WKU]	2	L4
L5	L1.clm. and l2	5	L5
L6	neri.in.	0	L6
L7	neri.in.	884	L7
L8	L7 and fibronect$	7	L8

END OF SEARCH HISTORY

WEST

Search Results - Record(s) 1 through 2 of 2 returned.

L3: Entry 1 of 2 File: USPT Jul 25, 2000

US-PAT-NO: 6093399
DOCUMENT-IDENTIFIER: US 6093399 A
**** See image for Certificate of Correction ****

TITLE: Methods and compositions for the specific coagulation of vasculature

DATE-ISSUED: July 25, 2000

US-CL-CURRENT: 424/182.1; 424/178.1, 424/179.1, 424/180.1, 530/387.1, 530/387.3, 530/387.7, 530/387.9, 530/388.1, 530/388.22, 530/388.85, 530/391.7, 530/391.9

INT-CL: [07] A61 K 39/395

L3: Entry 2 of 2 File: DWPI Jul 25, 2000

DERWENT-ACC-NO: 2000-531471
ABSTRACTED-PUB-NO: US 6093399A
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TITLE: New immunological and growth factor-based bispecific binding ligands, useful for stimulating coagulation in vasculature-associated diseases, e.g. for treating both benign and malignant diseases (e.g. meningioma or hemangioma)

INT-CL (IPC): A61 K 39/395
Derwent-CL (DC): B04, D16
CPI Codes: B04-G01; B04-G21; B04-H02; B04-H06; B04-H19; B12-M07; B14-F08; B14-H01B; D05-H11A;

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Search Results - Record(s) 1 through 2 of 2 returned.

L4: Entry 1 of 2 File: USPT Apr 18, 2000

US-PAT-NO: 6051230
DOCUMENT-IDENTIFIER: US 6051230 A
**** See image for Certificate of Correction ****

TITLE: Compositions for targeting the vasculature of solid tumors

DATE-ISSUED: April 18, 2000

US-CL-CURRENT: 424/178.1; 424/179.1, 424/180.1, 424/181.1, 424/182.1, 424/183.1, 530/387.1, 530/387.7, 530/388.1, 530/388.2

INT-CL: [07] A61 K 39/395, C07 K 16/00

L4: Entry 2 of 2 File: DWPI Apr 18, 2000

DERWENT-ACC-NO: 2000-363766
ABSTRACTED-PUB-NO: US 6051230A

TITLE: Novel compound for treating solid tumors comprising targeting agent that binds to tumor-associated endothelial cell marker expressed or localized on cell surface of intratumoral blood vessels and therapeutic agent

INT-CL (IPC): A61 K 39/395, C07 K 16/00
Derwent-CL (DC): B04, B05 , D16 , K08
CPI Codes: B02-R; B04-G02; B04-G04; B04-L01; B04-N01; B04-N02; B04-N03; B04-N04; B12-K04A1; B12-K04B; B14-H01; D05-H09; D05-H11; K09-B; K09-E;

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L2: Entry 27 of 73 File: USPT Mar 14, 2000

US-PAT-NO: 6036955
DOCUMENT-IDENTIFIER: US 6036955 A
**** See image for Certificate of Correction ****

TITLE: Kits and methods for the specific coagulation of vasculature

DATE-ISSUED: March 14, 2000

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Thorpe; Philip E.	Dallas	TX		
Edgington; Thomas S.	La Jolla	CA		

US-CL-CURRENT: 424/136.1; 424/130.1, 424/141.1, 424/143.1, 424/144.1, 424/145.1, 424/152.1, 424/155.1, 424/156.1, 424/158.1, 424/178.1, 424/85.2, 514/8, 530/387.3, 530/387.7, 530/388.7, 530/389.6, 530/389.7, 530/391.7

CLAIMS:

What is claimed is:

1. A kit comprising, in suitable container means:

(a) a first pharmaceutical composition comprising a biological agent capable of inducing the expression of an inducible marker in the intratumoral vasculature or stroma of a vascularized tumor; and

(b) a second pharmaceutical composition comprising a binding ligand that comprises a first binding region that binds to said inducible marker of intratumoral vasculature or stroma; the first binding region operatively linked to a coagulation factor or a second binding region that binds to a coagulation factor.

2. The kit of claim 1, wherein said first pharmaceutical composition comprises a biological agent capable of inducing the expression of a cytokine-inducible marker in the intratumoral vasculature or stroma of a vascularized tumor.

3. The kit of claim 1, wherein said first pharmaceutical composition comprises a biological agent capable of inducing the expression of a coagulant-inducible marker in the intratumoral vasculature or stroma of a vascularized tumor.

4. The kit of claim 1, wherein said first pharmaceutical composition comprises a biological agent capable of inducing the expression of an inducible marker in the

intratumoral stroma of a vascularized tumor.

5. The kit of claim 1, wherein said first pharmaceutical composition comprises a biological agent capable of inducing the expression of an inducible marker in the intratumoral vasculature of a vascularized tumor.

6. The kit of claim 5, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to an activating antigen on the cell surface of a leukocyte cell and to a tumor antigen on the cell surface of a tumor cell, the bispecific antibody being effective to induce the expression of a cytokine by a leukocyte cell in a vascularized tumor.

7. The kit of claim 6, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to the tumor cell antigen p185.sup.HER2, milk mucin core protein, TAG-72, Lewis a, carcinoembryonic antigen (CEA) or a tumor-associated antigen that binds to an antibody selected from the group consisting of B3 (ATCC HB 10573), 260F9 (ATCC HB 8488), D612 (ATCC HB 9796) and KS1/4, said KS1/4 antibody obtained from a cell comprising the vectors pGKC2310 (NRRL B-18356) and pG2A52 (NRRL B-18357).

8. The kit of claim 6, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to an activating antigen on the cell surface of a monocyte, macrophage, mast cell, helper T cell, CD8-positive T cell or NK cell.

9. The kit of claim 6, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to the activating antigen CD2, CD3, CD14, CD16 (FcR for IgE), CD28 or the T cell receptor antigen.

10. The kit of claim 6, wherein said first pharmaceutical composition comprises a bispecific antibody that induces the expression of the cytokine IL-1, TNF-.alpha., IFN-.gamma., IL-4 or TNF-.beta. by said leukocyte cell.

11. The kit of claim 6, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to the cytokine-inducible marker VCAM-1, E-selectin, endoglin, ICAM-1 or an MHC Class II antigen.

12. The kit of claim 6, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to the activating antigen CD14 and induces the expression of IL-1 by monocyte/macrophage cells.

13. The kit of claim 12, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to the activating antigen CD14 and to the tumor-associated antigen that binds to the antibody KS1/4, said KS1/4 antibody obtained from a cell comprising the vectors pGKC2310 (NRRL B-18356) and pG2A52 (NRRL B-18357).

14. The kit of claim 12, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to the cytokine-inducible marker VCAM-1.

15. The kit of claim 12, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to the cytokine-inducible marker E-selectin.

16. The kit of claim 12, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to the cytokine-inducible marker endoglin.

17. The kit of claim 6, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to CD28 and induces the expression of IFN-.gamma. by T cells.

18. The kit of claim 17, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to the

cytokine-inducible marker, an MHC Class II antigen.

19. The kit of claim 18, further comprising a third pharmaceutical composition comprising an agent capable of suppressing MHC Class II antigen expression in the vascular endothelial cells of normal tissues.

20. The kit of claim 19, wherein said third pharmaceutical composition comprises a cyclosporin.

21. The kit of claim 5, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to a tumor cell, a component of tumor vasculature or a component of tumor stroma, the first binding region operatively linked to a coagulation factor or a second binding region that binds to a coagulation factor; said antibody being effective to induce thrombin deposition within a tumor.

22. The kit of claim 21, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to a tumor cell.

23. The kit of claim 22, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to the tumor cell antigen p185.sup.HER2, milk mucin core protein, TAG-72, Lewis a, carcinoembryonic antigen (CEA) or a tumor-associated antigen that binds to an antibody selected from the group consisting of B3 (ATCC HB 10573), 260F9 (ATCC HB 8488), D612 (ATCC HB 9796) and KS1/4, said KS1/4 antibody obtained from a cell comprising the vectors pGKC2310 (NRRL B-18356) and pG2A52 (NRRL B-18357).

24. The kit of claim 21, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to a component of tumor stroma.

25. The kit of claim 24, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to a tumor-associated basement membrane component.

26. The kit of claim 24, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to VEGF within the tumor stroma.

27. The kit of claim 21, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to a component of tumor vasculature.

28. The kit of claim 27, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to a component expressed by a tumor vascular endothelial cell.

29. The kit of claim 28, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to VCAM-1, E-selectin, P-selectin, a VEGF receptor or endoglin.

30. The kit of claim 27, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to a ligand or growth factor that binds to a tumor vasculature cell surface receptor.

31. The kit of claim 30, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to VEGF or FGF.

32. The kit of claim 27, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to a ligand:receptor complex or a growth factor:receptor complex, but does not bind to the ligand or growth factor or to the receptor when the ligand or growth factor or the receptor is not in the ligand:receptor or the growth factor:receptor complex.

33. The kit of claim 21, wherein said first pharmaceutical composition comprises

an antibody comprising a first binding region operatively linked to a coagulation factor.

34. The kit of claim 21, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region operatively linked to a second binding region that binds to a coagulation factor.

35. The kit of claim 21, wherein the coagulation factor in said first pharmaceutical composition is Tissue Factor, a Tissue Factor derivative, prothrombin, Factor VII/VIIa, Factor IX/IXa, Factor X/Xa, Factor XI/XIa or Russell's viper venom Factor X activator.

36. The kit of claim 21, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to the coagulant-inducible marker E-selectin, P-selectin, PDGF or ICAM-1.

37. The kit of claim 36, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to the coagulant-inducible marker E-selectin.

38. The kit of claim 36, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to the coagulant-inducible marker P-selectin.

39. The kit of claim 1, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region operatively linked to a coagulation factor.

40. The kit of claim 1, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region operatively linked to a second binding region that binds to a coagulation factor.

41. The kit of claim 1, wherein the coagulation factor in said second pharmaceutical composition is Factor II/IIa, Factor VII/VIIa, Factor IX/IXa, Factor X/Xa, a vitamin K-dependent coagulation factor that lacks the Gla modification, Tissue Factor, a truncated Tissue Factor, a mutant Tissue Factor, a dimeric Tissue Factor, Russell's viper venom Factor X activator, thromboxane A.sub.2 or .alpha.2-antiplasmin.

42. The kit of claim 41, wherein the coagulation factor in said second pharmaceutical composition is Tissue Factor, a truncated Tissue Factor, a mutant Tissue Factor deficient in the ability to activate Factor VII or a dimeric Tissue Factor.

43. The kit of claim 1, further comprising an anti-tumor cell antibody-therapeuticagent conjugate.

44. A kit comprising, in suitable container means:

(a) a first pharmaceutical composition comprising a biological agent capable of inducing the expression of an inducible marker in the intratumoral vasculature of a vascularized tumor; and

(b) a second pharmaceutical composition comprising a binding ligand that comprises a first binding region that binds to said inducible marker of intratumoral vasculature; the first binding region operatively linked to a coagulation factor or a second binding region that binds to a coagulation factor.

45. The kit of claim 44, comprising:

(a) a first pharmaceutical composition comprising a bispecific antibody capable of inducing the expression of a cytokine-inducible marker in the intratumoral vasculature of a vascularized tumor, the bispecific antibody binding to a tumor cell surface antigen and to a leukocyte cell surface activation antigen and being effective to induce cytokine expression by a leukocyte cell; and

(b) a second pharmaceutical composition comprising a binding ligand that comprises a first binding region that binds to said cytokine-inducible marker of intratumoral vasculature; the first binding region operatively linked to a coagulation factor or a second binding region that binds to a coagulation factor.

46. The kit of claim 44, comprising:

(a) a first pharmaceutical composition comprising an antibody capable of inducing the expression of a coagulant-inducible marker in the intratumoral vasculature of a vascularized tumor, the antibody binding to a tumor cell, tumor stroma or tumor vascular antigen and to a coagulation factor and being effective to induce thrombin deposition within a tumor; and

(b) a second pharmaceutical composition comprising a binding ligand that comprises a first binding region that binds to said coagulant-inducible marker of intratumoral vasculature; the first binding region operatively linked to a coagulation factor or a second binding region that binds to a coagulation factor.

47. A kit comprising, in suitable container means:

(a) a first pharmaceutical composition comprising a first antibody that comprises a first binding region that binds to a component of the intratumoral vasculature or stroma of a vascularized tumor, the first binding region operatively linked to a coagulation factor or a second binding region that binds to a coagulation factor; and

(b) a second pharmaceutical composition comprising a second antibody comprising a distinct first binding region that binds to a distinct component of the intratumoral vasculature or stroma of a vascularized tumor, the distinct first binding region operatively linked to a coagulation factor or a second binding region that binds to a coagulation factor.

48. The kit of claim 47, wherein said first or second pharmaceutical compositions comprise an antibody comprising a first binding region that binds to VCAM-1, E-selectin, endoglin, ICAM-1, an MHC Class II antigen, VEGF or FGF.

49. The kit of claim 47, wherein said first pharmaceutical composition comprises an anti-VEGF-Tissue Factor antibody; and wherein said second pharmaceutical composition comprises an anti-VCAM-1-Tissue Factor antibody or an anti-E-selectin-Tissue Factor antibody.

50. A method for treating an animal having a vascularized tumor, the method comprising the steps of:

(a) introducing into the bloodstream of the animal a first pharmaceutical composition comprising a biological agent effective to induce the expression of an inducible marker in the intratumoral vasculature or stroma of a vascularized tumor; and

(b) introducing into the bloodstream of the animal a biologically effective amount of a second pharmaceutical composition comprising a binding ligand that comprises a first binding region that binds to said inducible marker of intratumoral vasculature or stroma; the first binding region operatively linked to a coagulation factor or a second binding region that binds to a coagulation factor.

51. The method of claim 50, wherein said first pharmaceutical composition comprises a biological agent effective to induce the expression of a cytokine-inducible marker in the intratumoral vasculature or stroma of a vascularized tumor.

52. The method of claim 50, wherein said first pharmaceutical composition comprises a biological agent effective to induce the expression of a coagulant-inducible marker in the intratumoral vasculature or stroma of a

vascularized tumor.

53. The method of claim 50, wherein said first pharmaceutical composition comprises a biological agent effective to induce the expression of an inducible marker in the intratumoral stroma of a vascularized tumor.

54. The method of claim 50, wherein said first pharmaceutical composition comprises a biological agent effective to induce the expression of an inducible marker in the intratumoral vasculature of a vascularized tumor.

55. The method of claim 54, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to an activating antigen on the cell surface of a leukocyte cell and to a tumor antigen on the cell surface of a tumor cell, the bispecific antibody being effective to induce the expression of a cytokine by a leukocyte cell in said vascularized tumor.

56. The method of claim 55, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to the tumor cell antigen p185.sup.HER2, milk mucin core protein, TAG-72, Lewis a, carcinoembryonic antigen (CEA) or a tumor-associated antigen that binds to an antibody selected from the group consisting of B3 (ATCC HB 10573), 260F9 (ATCC HB 8488), D612 (ATCC HB 9796) and KS1/4, said KS1/4 antibody obtained from a cell comprising the vectors pGKC2310 (NRRL B-18356) and pG2A52 (NRRL B-18357).

57. The method of claim 55, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to an activating antigen on the cell surface of a monocyte, macrophage, mast cell, helper T cell, CD8-positive T cell or NK cell.

58. The method of claim 55, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to the activating antigen CD2, CD3, CD14, CD16 (FcR for IgE), CD28 or the T cell receptor antigen.

59. The method of claim 55, wherein said first pharmaceutical composition comprises a bispecific antibody that induces the expression of the cytokine IL-1, TNF-.alpha., IFN-.gamma., IL-4 or TNF-.beta. by said leukocyte cell.

60. The method of claim 55, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to the cytokine-inducible marker VCAM-1, E-selectin, endoglin, ICAM-1 or an MHC Class II antigen.

61. The method of claim 55, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to the activating antigen CD14 and induces the expression of IL-1 by monocyte/macrophage cells.

62. The method of claim 61, wherein said first pharmaceutical composition comprises a bispecific antibody that binds to the activating antigen CD14 and to the tumor-associated antigen that binds to the antibody KS1/4, said KS1/4 antibody obtained from a cell comprising the vectors pGKC2310 (NRRL B-18356) and pG2A52 (NRRL B-18357).

63. The method of claim 61, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to the cytokine-inducible marker VCAM-1.

64. The method of claim 61, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to the cytokine-inducible marker E-selectin.

65. The method of claim 61, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to the cytokine-inducible marker endoglin.

66. The method of claim 55, wherein said first pharmaceutical composition

comprises a bispecific antibody that binds to CD28 and induces the expression of IFN-.gamma. by T cells.

67. The method of claim 66, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to the cytokine-inducible marker, an MHC Class II antigen.

68. The method of claim 66, wherein MHC Class II molecule expression by endothelial cells in the normal tissues of the animal is suppressed by cyclosporin administration, and MHC Class II molecule expression by endothelial cells in the intratumoral vasculature is induced by administration of said first pharmaceutical composition.

69. The method of claim 54, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to a tumor cell, a component of tumor vasculature or a component of tumor stroma, the first binding region operatively linked to a coagulation factor or a second binding region that binds to a coagulation factor; said antibody being effective to induce thrombin deposition within a tumor.

70. The method of claim 69, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to a tumor cell.

71. The method of claim 70, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to the tumor cell antigen p185.sup.HER2, milk mucin core protein, TAG-72, Lewis a, carcinoembryonic antigen (CEA) or a tumor-associated antigen that binds to an antibody selected from the group consisting of B3 (ATCC HB 10573), 260F9 (ATCC HB 8488), D612 (ATCC HB 9796) and KS1/4, said KS1/4 antibody obtained from a cell comprising the vectors pGKC2310 (NRRL B-18356) and pG2A52 (NRRL B-18357).

72. The method of claim 69, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to a component of tumor stroma.

73. The method of claim 72, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to a tumor-associated basement membrane component.

74. The method of claim 72, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to VEGF within the tumor stroma.

75. The method of claim 69, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to a component of tumor vasculature.

76. The method of claim 75, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to a component expressed by a tumor vascular endothelial cell.

77. The method of claim 76, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to VCAM-1, E-selectin, P-selectin, a VEGF receptor or endoglin.

78. The method of claim 75, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to a ligand or growth factor that binds to a tumor vascular endothelial cell surface receptor.

79. The method of claim 78, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region that binds to VEGF or FGF.

80. The method of claim 75, wherein said first pharmaceutical composition

comprises an antibody comprising a first binding region that binds to a ligand:receptor complex or a growth factor:receptor complex, but does not bind to the ligand or growth factor or to the receptor when the ligand or growth factor or the receptor is not in the ligand:receptor or the growth factor:receptor complex.

81. The method of claim 69, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region operatively linked to a coagulation factor.

82. The method of claim 69, wherein said first pharmaceutical composition comprises an antibody comprising a first binding region operatively linked to a second binding region that binds to a coagulation factor.

83. The method of claim 69, wherein the coagulation factor in said first pharmaceutical composition is Tissue Factor, a Tissue Factor derivative, prothrombin, Factor VII/VIIa, Factor IX/IXa, Factor X/Xa, Factor XI/XIa or Russell's viper venom Factor X activator.

84. The method of claim 69, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to the coagulant-inducible marker E-selectin, P-selectin, PDGF or ICAM-1.

85. The method of claim 84, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to the coagulant-inducible marker E-selectin.

86. The method of claim 84, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region that binds to the coagulant-inducible marker P-selectin.

87. The method of claim 50, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region operatively linked to a coagulation factor.

88. The method of claim 50, wherein said second pharmaceutical composition comprises a binding ligand that comprises a first binding region operatively linked to a second binding region that binds to a coagulation factor.

89. The method of claim 50, wherein the coagulation factor in said second pharmaceutical composition is Factor II/IIa, Factor VII/VIIa, Factor IX/IXa, Factor X/Xa, a vitamin K-dependent coagulation factor that lacks the Gla modification, Tissue Factor, a truncated Tissue Factor, a mutant Tissue Factor, a dimeric Tissue Factor, Russell's viper venom Factor X activator, thromboxane A.sub.2 or .alpha.2-antiplasmin.

90. The method of claim 89, wherein the coagulation factor in said second pharmaceutical composition is Tissue Factor, a truncated Tissue Factor, a mutant Tissue Factor deficient in the ability to activate Factor VII or a dimeric Tissue Factor.

91. The method of claim 50, further comprising administering to said animal an anti-tumor cell antibody-therapeuticagent conjugate.

92. The method of claim 50, wherein the animal is a human cancer patient.

93. A method for treating an animal having a vascularized tumor, the method comprising the steps of:

(a) introducing into the bloodstream of the animal a first pharmaceutical composition comprising a biological agent effective to induce the expression of an inducible marker in the intratumoral vasculature of a vascularized tumor; and

(b) introducing into the bloodstream of the animal a biologically effective amount of a second pharmaceutical composition comprising a binding ligand that

comprises a first binding region that binds to said inducible marker of intratumoral vasculature; the first binding region operatively linked to a coagulation factor or a second binding region that binds to a coagulation factor.

94. The method of claim 93, comprising the steps of:

(a) introducing into the bloodstream of the animal a first bispecific antibody, said bispecific antibody binding to both an activating antigen on the cell surface of a leukocyte and to a tumor cell surface antigen, the bispecific antibody being effective to induce the expression of a cytokine by leukocytes in the tumor; and

(b) introducing into the animal's bloodstream a biologically effective amount of a second antibody operatively linked to a coagulation factor or a second binding region that binds a coagulation factor, the second antibody binding to an antigen that is induced on the surface of intratumoral blood vessels of the vascularized tumor by said cytokine.

95. The method of claim 93, comprising the steps of:

(a) introducing into the bloodstream of the animal a first antibody, said antibody binding to a coagulation factor and to a tumor cell, tumor stroma or tumor vascular antigen of the tumor, the antibody being effective to induce the deposition of thrombin within the tumor; and

(b) introducing into the animal's bloodstream a biologically effective amount of a second antibody operatively linked to a coagulation factor or a second binding region that binds a coagulation factor, the second antibody binding to an antigen that is induced on the surface of intratumoral blood vessels of the vascularized tumor by the thrombin deposition.

96. A method for treating an animal having a vascularized tumor, the method comprising the steps of:

(a) introducing into the bloodstream of the animal a first antibody that comprises a first binding region that binds to a component of the intratumoral vasculature or stroma of a vascularized tumor, the first binding region operatively linked to a coagulation factor or a second binding region that binds to a coagulation factor; and

(b) introducing into the bloodstream of the animal a second antibody comprising a distinct first binding region that binds to a distinct component of the intratumoral vasculature or stroma of a vascularized tumor, the distinct first binding region operatively linked to a coagulation factor or a second binding region that binds to a coagulation factor.

97. The method of claim 96, wherein said first or second antibody comprises a first binding region that binds to VCAM-1, E-selectin, endoglin, ICAM-1, an MHC Class II antigen, VEGF or FGF.

98. The method of claim 96, wherein said first antibody is an anti-VEGF-Tissue Factor antibody; and wherein said second antibody is an anti-VCAM-1-Tissue Factor antibody or an anti-E-selectin-Tissue Factor antibody.

99. A method for treating an animal having a vascularized tumor, the method comprising the steps of:

(a) administering to said animal a biological agent effective to suppress the expression of MHC Class II molecules by endothelial cells in the normal tissues of the animal;

(b) administering to said animal a biological agent effective to induce the specific expression of MHC Class II molecules in the intratumoral vasculature of the vascularized tumor; and

(c) administering to said animal an antibody that binds to the induced MHC Class II molecules, the antibody linked to a coagulation factor or a second binding region that binds a coagulation factor.

100. The method of claim 99, wherein:

(a) the agent that suppresses MHC Class II molecule expression in the normal tissues is a cyclosporin; and

(b) the agent that induces MHC Class II molecule expression in the intratumoral vasculature of the vascularized tumor is a bispecific antibody that binds to a tumor cell antigen and to CD28 on the cell surface of a T cell, and induces IFN-.gamma. expression by said T cell.

101. The method of claim 99, wherein:

(a) the agent that suppresses MHC Class II molecule expression in the normal tissues is an anti-CD4 antibody that suppresses IFN-.gamma. production by the T cells of the animal; and

(b) the agent that induces MHC Class II molecule expression in the intratumoral vasculature of the vascularized tumor is an IFN-.gamma.-producing T cell clone that binds to a tumor antigen of the vascularized tumor.

102. The method of claim 101, wherein the IFN-.gamma.-producing T cell clone is prepared by a method comprising the steps of:

(a) removing a tissue section from the vascularized tumor of the animal;

(b) extracting infiltrating leukocytes from the tissue section; and

(c) expanding the infiltrating leukocytes in vitro to provide the IFN-.gamma. producing clone.

STIC-ILL

From: Portner, Ginny
Sent: Tuesday, August 19, 2003 4:25 PM
To: STIC-ILL
Subject: 09/512,082

Art Unit 1645

Thorpe et al., "Selective Killing of Proliferating Vascular Endothelial Cells by an Anti-Fibronectin Receptor Immunotoxin," 16th LH Gray Conference, University of Manchester Institute of Science and Technology, Sep. 17-21, 1990.

Other Reference Publication (89):
Aoyagi, "Distribution of Plasma Fibronectin in the Metastatic Lesion of Cancer: Experimental Study by Autoradiography," Thrombosis Research, 49:265-75, 1988.

Other Reference Publication (96):
Burton-Wurster, et al., "Expression of the Ed B Fibronectin Isoform in Adult Human Articular Cartilage," Biochemical and Biophysical Research Communication 165(2): 782-87, 1989.

Other Reference Publication (98):
Carnemolla et al., "A Tumor-associated Fibronectin Isoform Generated by Alternative Splicing of Messenger RNA Precursors," The Journal of Cell Biology, 108:1139-48, 1989.

Other Reference Publication (99):
Carnemolla et al., "The Inclusion of the Type III Repeat ED-B in the Fibronectin Molecule Generates Conformational Modifications that Unmask a Cryptic Sequence," The Journal of Biological Chemistry, 267(34):245898-92, 1992.

Other Reference Publication (100):
Carnemolla, et al., "Phage Antibodies with Pan-Species Recognition of the Oncofoetal Angiogenesis Marker Fibronectin ED-B Domain," Int. J. Cancer 68:397-505, 1996.

Other Reference Publication (101):
Castellani, et al., "The Fibronectin Isoform Containing the ED-B Oncofoetal Domain: A Marker of Angiogenesis," Int. J. Cancer, 59:612-18, 1994.

Other Reference Publication (104):
Farnoud, et al., "Fibronectin Isoforms Are Differentially Expressed in Normal and Adenomatous Human Anterior Pituitaries," Int. J. Cancer, 61:27-34, 1995.

Other Reference Publication (108):
Kaczmarek, et al., "Distribution of Oncofetal Fibronectin Isoforms in Normal Hyperplastic and Neoplastic Human Breast Tissues," Int. J. Cancer, 58:11-16, 1994.

Other Reference Publication (112):
Linnala, et al., "Human Amnion Epithelial Cells Assemble Tenascins and Three Fibronectin Isoforms in the Extracellular Matrix," FEBS, 314(1,2):74-78, 1993.

Other Reference Publication (115):
Oyama, et al., "Coordinate Oncodevelopmental Modulation of Alternative Splicing of Fibronectin Pre-Messenger RNA at ED-A, ED-B and CS1 Regions in Human Liver Tumors," Cancer Research 53:2005-11, 1993.

Other Reference Publication (116):
Peters, et al., "Expression of the Alternatively Spliced EIIB Segment of Fibronectin," Cell Adhesion and Communication, 3:67-89, 1995.

Other Reference Publication (126):
Saiki et al., "Inhibition of Tumor Angiogenesis by a Synthetic Cell-Adhesive Polypeptide Containing the Arg-Gly-Asp (RGD) Sequence of Fibronectin, Poly(RGD)," Jpn. J. Cancer Res. 81:668-75, 1990.

Other Reference Publication (128):
Schwarzbauer, "Alternative Splicing of Fibronectin: Three Variants, Three Functions," BioEssays 13(10)527-33, 1991.

Other Reference Publication (133):
Ueda, et al., "Selective Distribution of Fibronectin to a Tumor-Cell Line," Cancer Letters 31:261-65, 1986.

Other Reference Publication (134):
Vartio, et al, "Differential Expression of the ED Sequence-Containing Form of Cellular Fibronectin in Embryonic and Adult Human Tissues," Journal of Cell Science, 88:419-30, 1987.

Int. J. Cancer: 59, 612–618 (1994)

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THE FIBRONECTIN ISOFORM CONTAINING THE ED-B ONCOFETAL DOMAIN: A MARKER OF ANGIOGENESIS

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Different fibronectin (FN) isoforms are generated by the alternative splicing of 3 regions (ED-A, ED-B and IIICS) of the primary transcript. The FN isoform containing the ED-B sequence, a complete type-III-homology repeat, while having extremely restricted distribution in normal adult tissues, reveals high expression in fetal and tumor tissues. Using the monoclonal antibody (MAb) BC-1, specific for the FN isoform containing the ED-B sequence (B^+ · FN), we demonstrate here, using immunohistochemical techniques, that while this FN isoform is undetectable in mature vessels, it is highly expressed during angiogenesis both in neoplastic and in normal tissues, as in the case of the functional layer of endometrium during the proliferative phase. B^+· FN is thus a marker for the formation of new vessels, and the BC-1 MAb may be a useful reagent for evaluating the level of the angiogenetic process in different neoplasms.

Fibronectins (FNs) are high-molecular-mass adhesive glycoproteins present in the extracellular matrix and in body fluids. These molecules are involved in various biological phenomena, such as the establishment and maintenance of normal cell morphology, cell migration, hemostasis and thrombosis, wound healing and oncogenic transformation (Alitalo and Vaheri, 1982; Ruoslahti, 1988; Yamada, 1983).

FN polymorphism is due to alternative splicing patterns in 3 regions (IIICS, ED-A and ED-B) of the single FN primary transcript (Fig. 1) (Hynes, 1985; Zardi *et al.*, 1987), as well as to post-translational modifications. The alternative splicing of the FN pre-mRNA is developmentally regulated and cell-type specific (Carnemolla *et al.*, 1989 ffrench-Constant and Hynes, 1989; Kornblihtt *et al.*, 1984; Norton and Hynes, 1987; Oyama *et al.*, 1989; Vartio *et al.*, 1987). Furthermore, it has been demonstrated that the splicing pattern of FN pre-mRNA is de-regulated in transformed cells and in malignancies (Zardi *et al.*, 1987; Carnemolla *et al.*, 1989; Vartio *et al.*, 1987; Borsi *et al.*, 1987; Castellani *et al.*, 1986; Oyama *et al.*, 1989, 1990). In fact, the FN isoforms containing the IIICS, ED-A and ED-B sequences are expressed to a greater degree in transformed human cells and in tumor tissues than in their normal counterparts. In particular, as established immunohistochemically using the BC-1 MAb specific for the FN isoform containing the ED-B sequence (B^+ · FN), while this isoform is undetectable in normal adult tissues, with some very rare exceptions, it shows much greater expression in the extracellular matrix of fetal and tumor tissues (Carnemolla *et al.*, 1989). We have therefore named the ED-B sequence "oncofetal domain".

The ED-B sequence is a complete type-III-homology repeat composed of 91 amino acids, is coded for by a single exon and is the most conserved FN region, with 100% and 96% homology with rat and chicken FN respectively (Zardi *et al.*, 1987; Norton and Hynes, 1987). This could indicate either a more recent evolution of the ED-B sequence, with less time to diverge, or a more stringent requirement due to some unknown function(s) performed by this sequence.

Using a MAb (BC-1) specific for the B^+ · FN isoform, we show immunohistochemically that this FN isoform is undetectable in mature vessels, while it is present during angiogenetic processes both in neoplastic and in normal tissues.

MATERIAL AND METHODS

Tissues

Normal and neoplastic tissues were obtained from samples taken during the course of therapeutic surgical procedures. The samples investigated included 47 tumors of the meninges, 33 glioblastomas and anaplastic astrocytomas; 10 low-grade astrocytomas, 3 ependymomas, 2 breast intraductal papillomas and 5 endometria. Each sample was divided into 2 parts: one was processed for conventional histopathological diagnosis and the other was immediately frozen in liquid nitrogen. Cryostat sections 5 μm thick were stained with hematoxylin-eosin, and additional frozen sections were used for immunohistochemical staining after fixation in absolute cold acetone for 10 min. To prevent the heterogeneous distribution of TN within the tissues from giving false-negative results, at least 3 non-consecutive sections of the biopsy were analyzed. Astrocytic tumors were graded and meninges tumors were classified according to Kleihues *et al.* (1993).

Monoclonal antibodies (MAbs)

The MAbs used were BC-1, which recognizes the B^+ · FN isoform; IST-6, which recognizes only fibronectin molecules without the ED-B sequence (B^- · FN); and IST-4, which is specific for all FN isoforms. The characterization of these antibodies has been reported by Carnemolla *et al.* (1989, 1992). The anti-human Von-Willebrand-factor MAb (Dako-Factor VIII) and the MAb specific for proliferating cells, KI-67, were purchased from Dako (Carpenteria, CA). The MAb anti-α-smooth-muscle-actin (α-sm1) (Skalli *et al.*, 1986) was a kind gift from Drs. Chaponnier and Gabbiani (Department of Pathology, University of Geneva, Switzerland).

Immunohistochemical procedures

For immunohistochemical studies, 5-μm-thick cryostat sections were air-dried and fixed in cold acetone for 10 min. Immunostaining was performed using a streptavidin-biotin-alkaline-phosphatase-complex staining kit (Bio-Spa Division, Milan, Italy) and naphtol-AS-MX-phosphate and Fast-Red TR (Sigma, St. Louis, MO) to visualize binding sites. Gill's hematoxylin was used as a counterstain, followed by mounting in glycergel (Dako).

Double-staining experiments were performed according to Sternberger and Shirley (1979). The first reaction sequence

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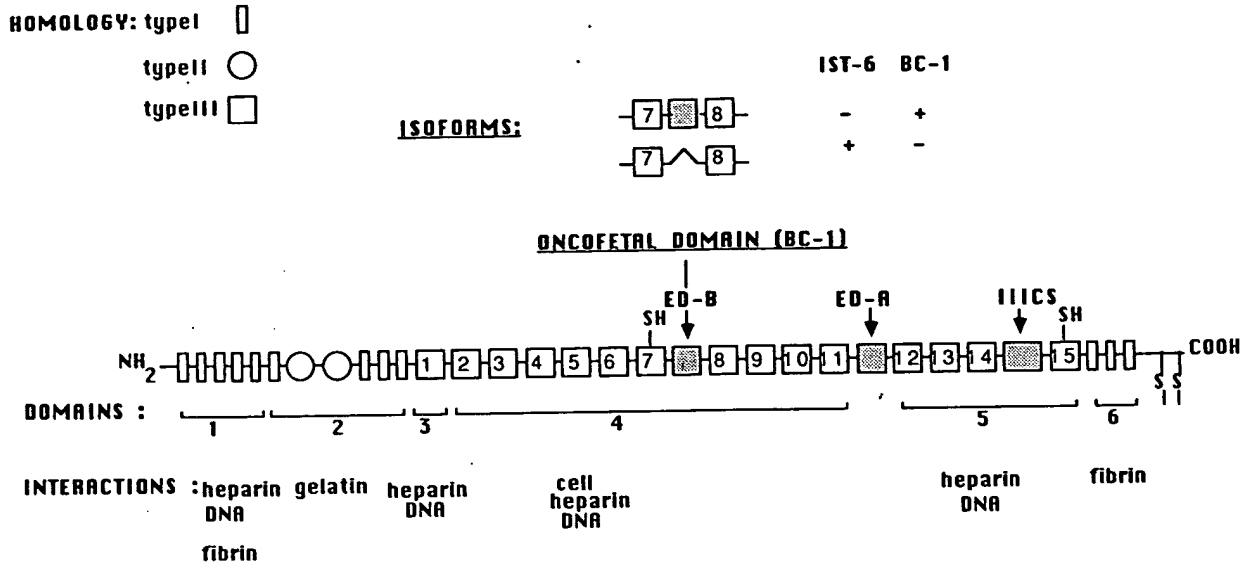

FIGURE 1 – – Model of the domain structure of a sub-unit of human fibronectin. The oncofetal domain ED-B is indicated. The figure also indicates the internal homologies, the 3 sites of alternative splicing (ED-B, ED-A and IIICS), the major macromolecules interacting with the various FN domains, the 2 isoforms generated by alternative splicing of the ED-B sequence, and the reactivity of these isoforms with the IST-6 and BC-1 MAbs. The characterization of BC-1 and IST-6 has been reported by Carnemolla *et al.* (1992), modified from Hynes (1985).

consisted in the application of the primary MAb, incubation with biotinylated goat anti-mouse IgG (Bio-Spa) and then with avidin-biotinylated-peroxidase complex (Bio-Spa). Immunoenzymatic staining was then carried out using 3,3-diaminobenzidine tetrahydrochloride (DAB)-H_2O_2 (Sigma), which yielded a brown reaction product masking antigens and immunoreagents of this first sequence and thus preventing cross-binding of the antibodies in the second sequence. Next, the sections were incubated with the second primary MAb, then incubated with the secondary antibody and streptavidin-biotinylated-alkaline-phosphatase complex (Bio-Spa). The red reaction product was obtained using a mixture of 2 mg naphtol AS-MX phosphate (Sigma) dissolved in 200 µl of n,n-dimethylformamide (Sigma) and diluted in 9.8 ml of 0.1 M Tris-HCl buffer, pH 8.2, and 1 mM levamisole (Sigma). Immediately before use, 10 mg of Fast-Red TR salt (Sigma) were added. Gill's hematoxylin was used as a counterstain, and the sections were mounted in glycergel (Dako).

RESULTS

We have extensively studied the distribution of this B^+ · FN isoform in the vessels of normal, regenerating and neoplastic tissues, using the BC-1 MAb specific for this FN isoform (Carnemolla *et al.*, 1989, 1992). In particular, we have studied the presence of B^+ · FN in the vessels of glioblastoma multiforme, since endothelial proliferation and angiogenetic processes, which lead to the formation of capillary clusters, are often very prominent in these neoplasms.

Figure 2*a* shows the absence of B^+ · FN in the blood vessels of normal adult cerebellum, while Figure 2*b* shows the presence in these serial sections of blood vessels highlighted using a MAb to factor VIII.

Figure 2*c* shows a section of glioblastoma multiforme with a typical capillary cluster double-stained with the BC-1 (red) and KI-67 (brown) MAbs. Note the hyperplasia of the endothelial cells, the intense cytoplasmatic staining of these cells by BC-1 and a number of nuclei of endothelial cells stained by KI-67, indicating endothelial-cell proliferation. On the contrary, neoplastic cells showed no reaction with BC-1. Similar results were obtained in all 33 cases of anaplastic astrocytoma and glioblastoma multiforme studied.

Figure 2*d* shows the same double-staining procedure, with the BC-1 and KI-67 MAbs, of a glioblastoma multiforme section containing both neoplastic tissue and a portion of neighboring normal cerebral cortex. It should be noted that, while the vessels are intensively stained by BC-1 in the neoplastic portion, the vessels of the adjacent normal brain tissue are negative. Staining using a MAb specific for α-smooth-muscle actin, a marker for pericytes, has demonstrated the absence of pericytes in the capillary clusters or vessels with hyperplastic endothelial cells in glioblastoma multiforme (Fig. 2*e*).

Figure 3*a,b* shows serial sections of a glomerular-like vascular structure of a glioblastoma multiforme stained with BC-1 *(a)* and with an anti-factor-VIII MAb *(b)*. BC-1 stained the cytoplasm of the endothelial cells. Figure 3*c* shows another glomerular-like vascular structure of a glioblastoma multiforme, double-stained using the anti-factor-VIII MAb (red) and BC-1 (brown). The 2 MAbs stained the same cells with preferential baso-lateral distribution of the B^+ · FN. Figure 3*d* shows double staining of a glioblastoma section with the BC-1 (brown) and IST-6 (red) MAbs, the latter being specific only for FN molecules not containing the sequence ED-B (B^- · FN). Only the capillary clusters or vessels with hyperplastic endothelial cells were stained by BC-1. On the contrary, in vessels with normal morphology, even though containing FN as shown by the reaction with IST-6, B^+ · FN was not detectable.

Only in 3 out of 10 cases of low-grade astrocytoma and in 16 out of 47 cases of meningioma, and in very few vessels of each specimen, was B^+ · FN detectable; on the other hand, no B^+ · FN was detectable in the remaining cases. Figure 4*a,b* show serial sections of meningothelial meningioma stained with the anti-factor-VIII MAb and with BC-1 respectively. It is possible

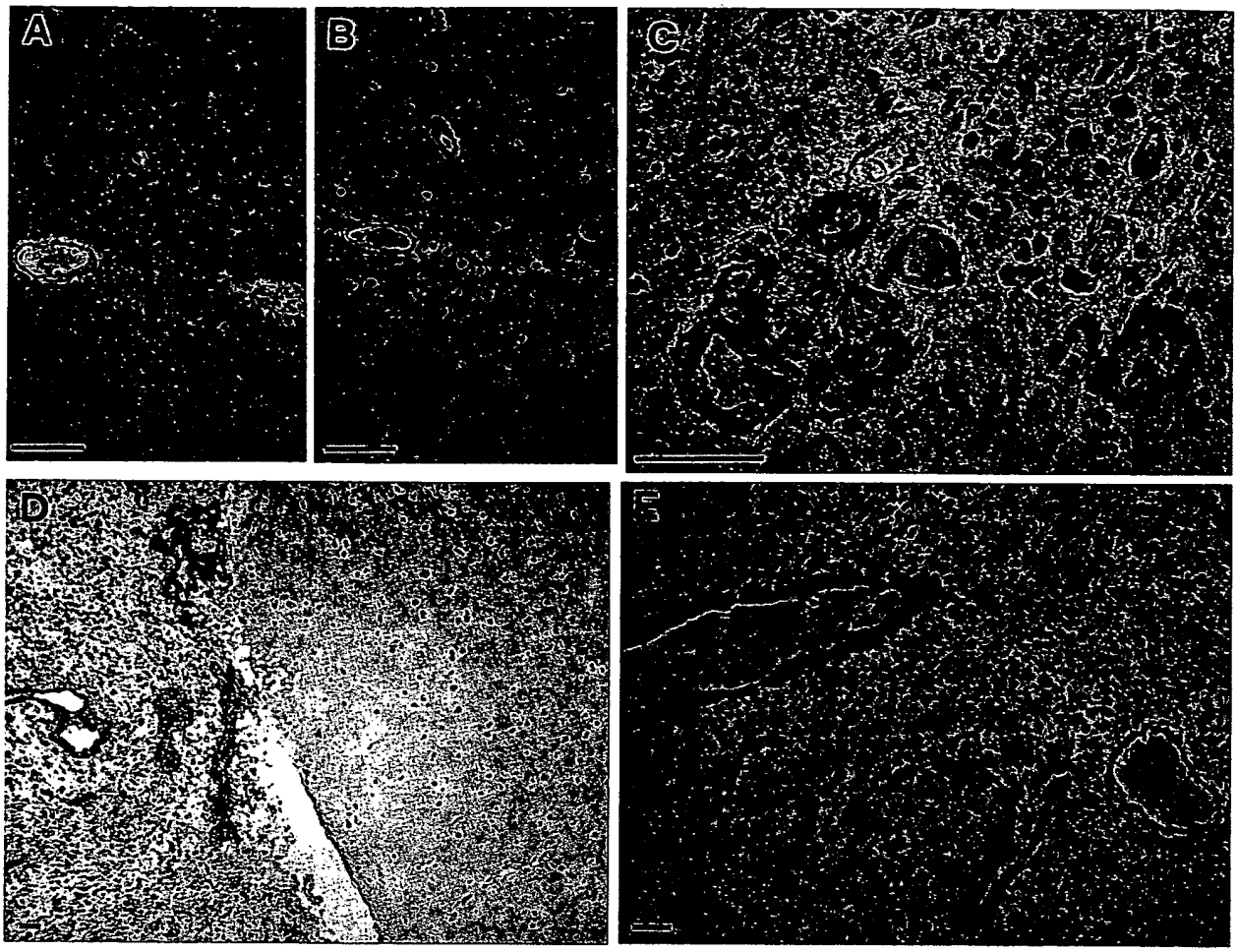

FIGURE 2 – Section of normal human cerebellum stained with a MAb specific for factor VIII *(a)* and with BC-1 *(b)*. *(c)* Section of a specimen of glioblastoma multiforme double stained using the KI-67 MAb (brown), specific for proliferating cells and BC-1 (red). BC-1 stained the endothelial cells of the glomerular-like vascular structures. Some endothelial cells actively proliferated. *(d)* Section of glioblastoma-multiforme specimen showing neoplastic and normal tissue stained with BC-1 (red) and with KI-67 (brown). Note the high number of proliferating cells in the neoplastic tissue. Only the vessels within the tumors were stained by the BC-1. *(e)* Section of a specimen of glioblastoma multiforme double stained with the α-sm 1 MAb (brown) specific for smooth-muscle actin and with BC-1 (red). Mature vessels reacted with the α-sm 1 MAb but not with BC-1, while BC-1-positive vessels did not react with α-sm 1. Scale bar, 10 μm.

to note that the vessels present in the sections did not react with BC-1. This absence of reaction cannot be attributed to a general lack of FN, as clearly shown in Figure 4*c,d* where 2 serial sections of a psammomatous meningioma are stained with BC-1 and with IST-6. While the section stained with BC-1 did not show any reaction, that stained with IST-6 showed a strong diffuse reaction, especially in the vessels. However, all the atypical and anaplastic meningiomas studied, which are characterized by a higher malignancy and frequent mitoses, showed the presence of $B^+ \cdot FN$ in a high percentage of blood vessels (data not shown), probably because angiogenetic processes are more pronounced in these tumors.

Since the data described above indicated a correlation between the presence of $B^+ \cdot FN$ and the angiogenetic processes, we studied the endometrium, a tissue in which angiogenesis occurs physiologically. Figure 5*a,b* shows 2 serial sections of the functional (superficial) layer of an endometrium in the proliferative phase, immunostained with BC-1 and with the anti-factor-VIII MAb respectively. In this part of endometrium, where proliferative and angiogenetic processes are present, BC-1 stained both the connective tissues and the vessels with a pattern similar to that observed in various carcinomas (Carnemolla *et al.*, 1989). Figure 5*c,d* shows the 2 serial sections, from the same specimens as in Figure 5*a,b* with the basal (deep) layer of endometrium and the myometrium immunostained using BC-1 and the anti-factor-VIII MAb respectively. There are no proliferative or angiogenetic processes in this area. BC-1 did not show any staining (Fig. 5*c*), while the anti-factor-VIII MAb showed the presence of a large number of vessels (Fig. 5*d*). Furthermore, endometria from post-menopausal subjects showed no reaction with BC-1 (data not shown).

DISCUSSION

Interactions between endothelial cells and various extracellular-matrix (ECM) components have been reported to play important roles during angiogenetic processes such as modula-

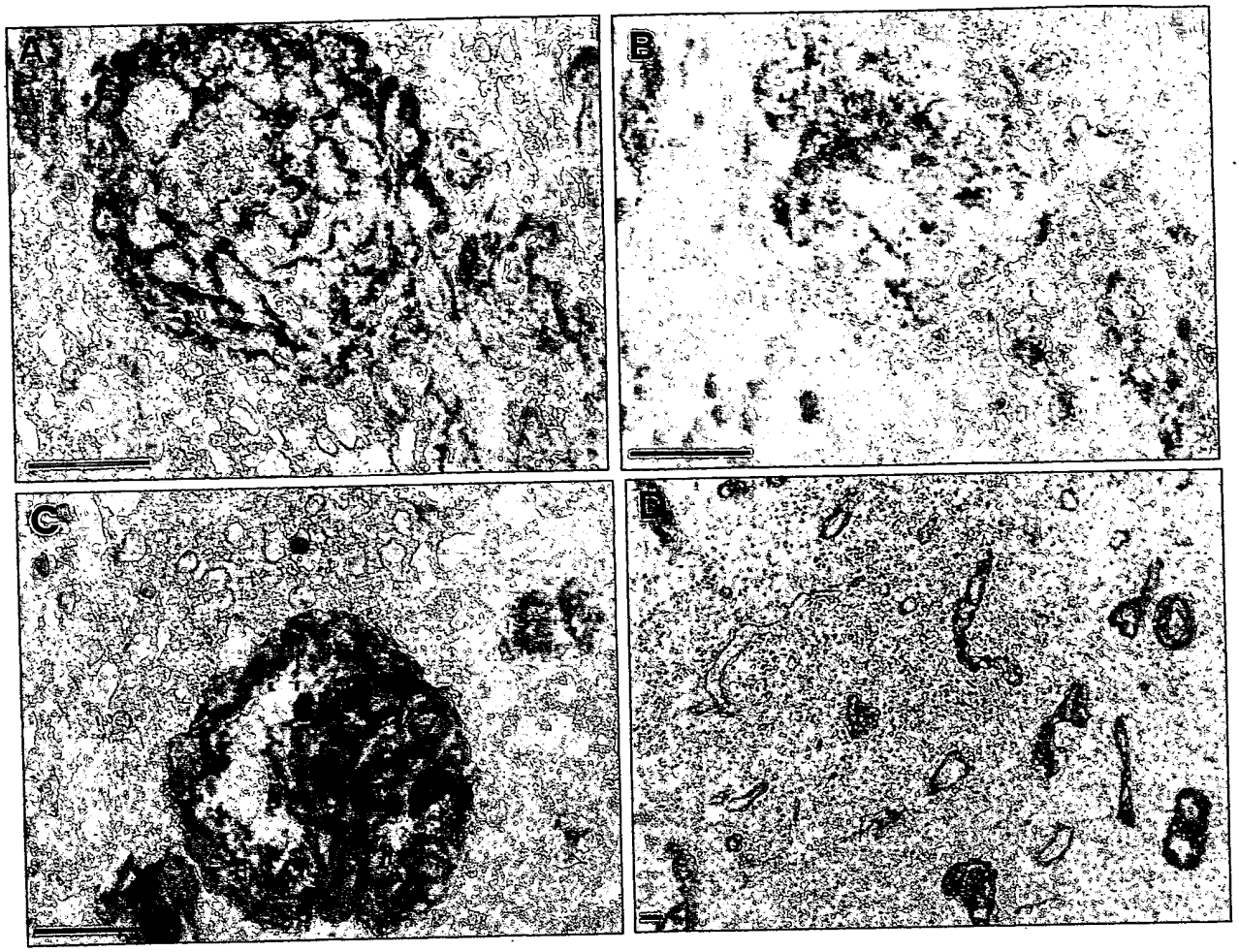

FIGURE 3 – Serial sections of glioblastoma multiforme showing a glomerular-like vascular structure stained with BC-1 *(a)* and the anti-factor-VIII MAb *(b)*. *(c)* Section of glioblastoma multiforme with a glomerular-like vascular structure double-stained with the anti-factor-VIII MAb (red) and with BC-1 (brown). Both MAbs stained endothelial cells with a preferential baso-lateral distribution of the B^+ · FN isoform. *(d)* Section of glioblastoma-multiforme double stained with BC-1 (brown), specific for B^+ · FN, and the IST-6 MAb (red), specific for B^- · FN. Pseudoglomerular structures reacted with the BC-1, while blood vessels with normal morphology did not show the presence of B^+ · FN, though they reacted intensively with the IST-6 MAb specific for FN molecules not containing the ED-B sequence. Scale bar, 10 μm.

tion of endothelial-cell migration and proliferation (Madri and Williams, 1983; Ingber and Folkman, 1989). It is known that one of the major components of the provisional ECM of growing blood microvessels is FN. In fact, Risau and Lemmon (1988) have shown that migrating and proliferating endothelial cells are associated with an FN-rich matrix, whereas mature capillaries have a laminin-containing basement membrane. Furthermore, these authors have shown that endothelial cells themselves, during migration and proliferation, produce the appropriate matrix which includes FN. These and our data disagree with those reported by Higuchi *et al.* (1993), which indicate that the degree of histological cell malignancy and de-differentiation of human gliomas are inversely correlated with the expression of FN in the blood vessels. The discrepancy between these observations and our findings may be due to the fact that Higuchi *et al.* (1993) used formalin-fixed and paraffin-embedded sections instead of cryostat sections. In fact, we have observed that the former fixation procedure greatly reduced the sensitivity of immunohistochemical reaction.

The B^+ · FN isoform has extremely restricted distribution in adult human tissues, while it is highly expressed in the ECM of the stroma of a variety of tumors and fetal tissues, as well as by macrophages and fibroblasts during cutaneous wound healing (Carnemolla *et al.*, 1989; Brown *et al.*, 1993). Carnemolla *et al.* (1989) have also documented the absence of B^+ · FN in the blood vessels of a large number of normal adult tissues, with the exception of a few vessels of the ovary, tissues in which, however, angiogenetic processes are present. Studying the distribution of the B^+ · FN isoform in glioblastoma multiforme, we have found that all the vessels with hyperplastic endothelial cells reacted strongly with BC-1, while vessels with monostratified non-hypertrophic endothelium did not react at all. These latter vessels, however, were not devoid of FN, as demonstrated using the IST-6 and IST-4 MAbs specific for B^- · FN and all FN isoforms respectively. Considering that angiogenetic processes are prominent in these neoplasia this observation suggested an association between angiogenesis and expression of the B^+ · FN.

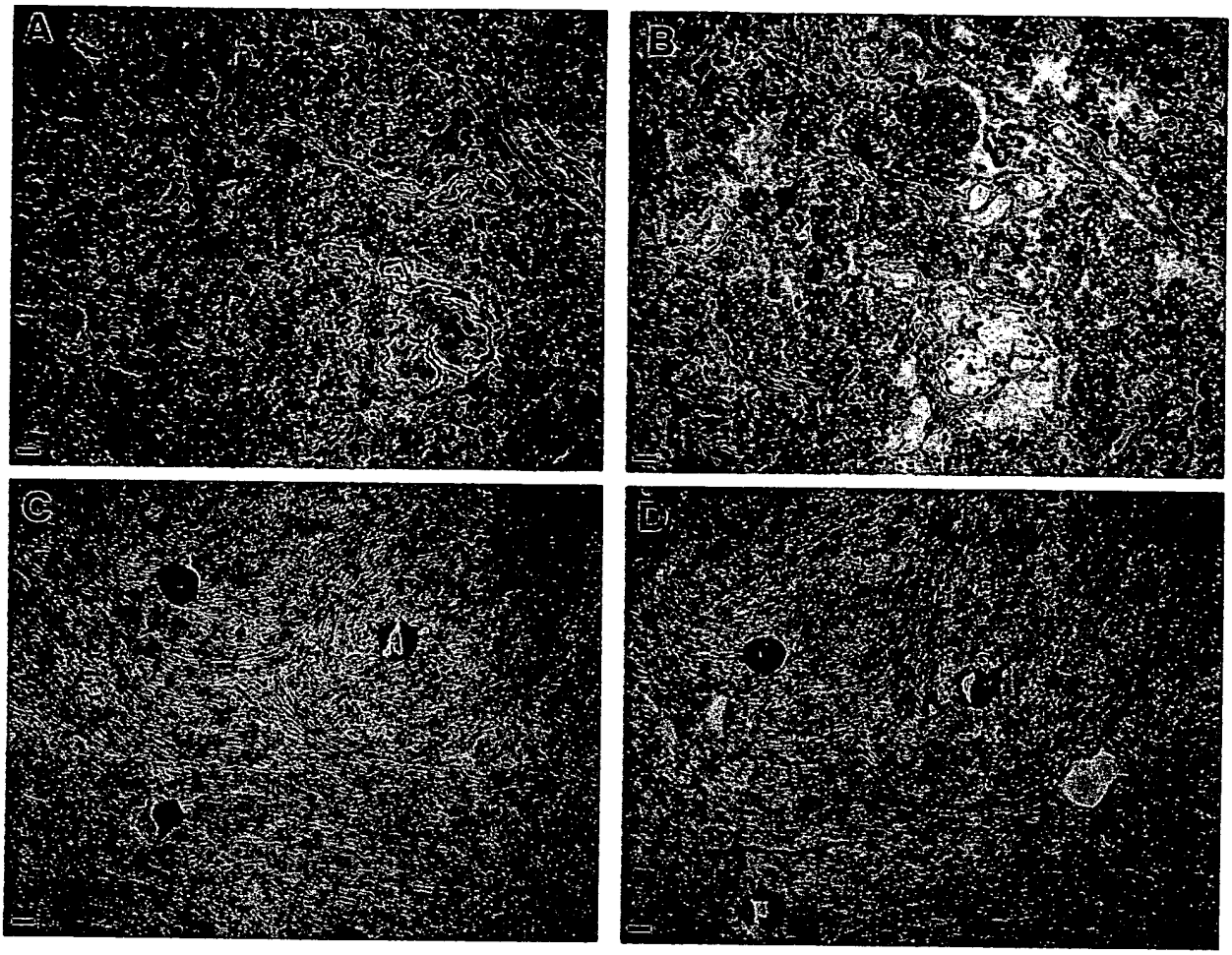

FIGURE 4 – *(a,b)* Serial sections of a meningothelial meningioma stained with the anti-factor-VIII MAb *(a)* and with BC-1 *(b)*. The blood vessels did not react with BC-1. *(c,d)* Serial sections of a psammomatous meningioma stained with IST-6 *(c)* and BC-1 *(d)*. Note that, even though IST-6 showed the presence of large amounts of FN, especially in the blood vessels, BC-1 showed no reaction. Scale bar, 10 μm.

A large number of BC-1-positive vessels were found not only in glioblastoma multiforme or high-grade astrocytoma but, regardless of the malignancy, also in benign tumors, such as ependimoma and breast intraductal papilloma, neoplasms with a considerable level of new blood-vessel formation (data not shown).

The association of B^+ · FN with angiogenesis has also been demonstrated by the study of endometria during the proliferative phase. This analysis showed a very strong reaction in the vessels of the functional layer, where angiogenetic processes are present, but not in the endometrial basal layer and in the myometrium, where angiogenesis is absent.

In conclusion, these data show a very high level of accumulation of the B^+ · FN isoform during angiogenesis. Our immunohistochemical data indicate that the isoform is produced by endothelial cells, given that a large amount of this FN isoform is localized within the endothelial-cell cytoplasm. *In situ* hybridization experiments will be necessary for definitive and unequivocal identification of the cell type responsible for B^+ · FN synthesis. However, regardless of the former point, the clear localization of B^+ · FN in newly forming blood vessels strongly suggests that it may be involved in the angiogenetic process and in endothelial-cell migration.

The BC-1 MAb has thus proved useful for evaluating the level of formation of new blood-vessels in different neoplasms, a process indicating malignancy in various tumors, *e.g.*, glioblastoma and breast cancer. BC-1, therefore, may be useful for prognosis, as well as for the differential diagnosis of meningioma *vs.* atypical and anaplastic meningioma and of astrocytoma *vs.* anaplastic astrocytoma and glioblastoma multiforme. The role of the B^+ · FN isoform during angiogenesis, as well as the clinical potential of BC-1 for prognostic, diagnostic and anti-angiogenic therapeutic purposes, are under investigation.

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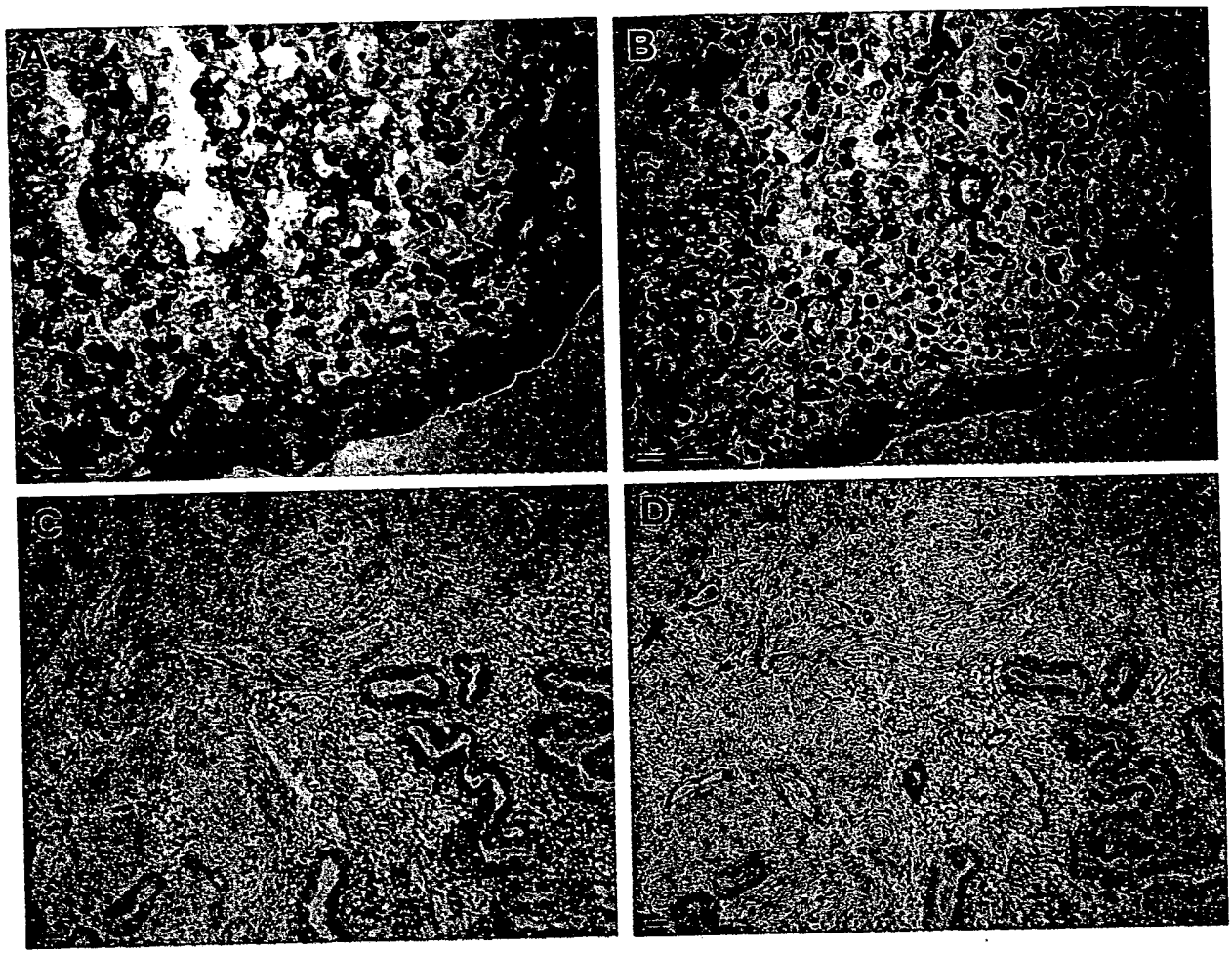

FIGURE 5 – Serial sections of an endometrium in the proliferative phase showing the functional (superficial) layer *(a,b)* and part of the myometrium and the endometrial basal layer *(c,d)* stained with BC-1 *(a,c)* and with the anti-factor-VIII MAb *(b,d)*. Note that the extracellular matrix and the vessels are stained by BC-1 only in the functional layer, whereas they are negative both in the endometrial basal layer and in the myometrium. Scale bar, 10 μm.

REFERENCES

ALITALO, K. and VAHERI, A., Pericellular matrix in malignant transformation. *Advanc. Cancer Res.*, **37**, 111–158 (1982).

BORSI, L., CARNEMOLLA, B., CASTELLANI, P., ROSELLINI, C., VECCHIO, D., ALLEMANNI, G., CHANG, S.E., TAYLOR-PAPADIMITRIOU, J., PANDE, H. and ZARDI, L., Monoclonal antibodies in the analysis of fibronectin isoforms generated by alternative splicing of mRNA precursors in normal and transformed human cells. *J. Cell Biol.*, **104**, 595–600 (1987).

BROWN, L.F., DUBIN, D., LAVIGNE, L., LOGAN, B., DVORAK, H.F. and VAN DE WATER, L., Macrophages and fibroblasts express embryonic fibronectins during cutaneous wound healing. *Amer. J. Pathol.*, **142**, 793–801 (1993).

CARNEMOLLA, B., BALZA, E., SIRI, A., ZARDI, L., NICOTRA, M.R., BIGOTTI, A. and NATALI, P.G., A tumor-associated fibronectin isoform generated by alternative splicing of messenger-RNA precursors. *J. Cell Biol.*, **108**, 1139–1148 (1989).

CARNEMOLLA, B., LEPRINI, A., ALLEMANNI, G., SAGINATI, M. and L. ZARDI., The inclusion of the type-III repeat ED-B in the fibronectin molecule generates conformational modifications that unmask a cryptic sequence. *J. biol. Chem.*, **267**, 24689–24692 (1992).

CASTELLANI, P., SIRI, A., ROSELLINI, C., INFUSINI, E., BORSI, L. and ZARDI, L., Transformed human cells release different fibronectin variants than do normal cells. *J. Cell Biol.*, **103**, 1671–1677 (1986).

FFRENCH-CONSTANT, C. and HYNES, R.O., Alternative splicing of fibronectin is temporally and spatially regulated in the chicken embryo. *Development*, **106**, 375–388 (1989).

HIGUCHI, M., OHNISHI, T., ARITA, N., HIRAGA, S. and HAYAKAWA, T., Expression of tenascin in human gliomas: its relation to histological malignancy, tumor differentiation and angiogenesis. *Acta neuropathol.*, **85**, 481–487 (1993).

HYNES, R.O., Molecular biology of fibronectin. *Ann. Rev. Cell Biol.*, **1**, 67–90 (1985).

INGBER, D. and FOLKMAN, J., How does extracellular matrix control capillary morphogenesis? *Cell*, **58**, 803–805 (1989).

KLEIHUES, P., BURGER, P.C. and SCHEITHAUER, B.W., *Histological typing of tumours of the central nervous system.* Springer, (1993).

KORNBLIHTT, A.R., VIBE-PEDERSEN, K. and BARALLE, F.E., Human fibronectin: cell-specific alternative mRNA splicing generates polypeptide chains differing in the number of internal repeats. *Nucleic Acids Res.*, **12**, 5853–5868 (1984).

MADRI, J.A. and WILLIAMS, S.K., Capillary endothelial cell cultures: phenotypic modulation by matrix components. *J. Cell Biol.*, **97**, 153–165 (1983).

NORTON, P.A. and HYNES, R.O., Alternative splicing of chicken fibronectin in embryos and in normal and transformed cells. *Mol. cell. Biol.*, **7**, 4297–4307 (1987).

OYAMA, F., HIROHASHI, S., SHIMOSATO, Y., TITANI, K. and SEKIGUCHI, K., Oncodevelopmental regulation of the alternative splicing of fibronectin pre-messenger RNA in human lung tissues. *Cancer Res.*, **50**, 1075–1078 (1990).

OYAMA, F., MURATA, Y., SUGANUMA, N., KIMURA, T., TITANI, K. and SEKIGUCHI, K., Patterns of alternative splicing of fibronectin pre-mRNA in human adult and fetal tissues. *Biochemistry*, **28**, 1428–1434 (1989).

RISAU, W. and LEMMON, V., Changes in the vascular extracellular matrix during embryonic vasculogenesis and angiogenesis. *Devel. Biol.*, **125**, 441–450 (1988).

RUOSLAHTI, E., Fibronectin and its receptors. *Ann. Rev. Biochem.*, **57**, 375–413 (1988).

SKALLI, O., ROPRAZ, P., TRZECIAK, A., BENZONANA, G., GILLESSEN, D. and GABBIANI, G., A monoclonal antibody against α smooth-muscle actin: a new probe for smooth-muscle differentiation. *J. Cell Biol.*, **103**, 2787–2796 (1986).

STERNBERGER, L.A. and SHIRLEY, A.J., The unlabeled-antibody method. *J. Histochem. Cytochem.*, **27**, 1424–1429 (1979).

VARTIO, T., LAITINEN, L., NARVANEN, O., CUTOLO, M., THORNELL, L.E., ZARDI, L. and VIRTANEN, I., Differential expression of the ED-sequence-containing form of cellular fibronectin in embryonic and adult human tissues. *J. Cell Sci.*, **88**, 419–430 (1987).

YAMADA, K.M., Cell-surface interactions with extracellular materials. *Ann. Rev. Biochem.*, **52**, 761–799 (1983).

ZARDI, L., CARNEMOLLA, B., SIRI, A., PETERSEN, T.E., PAOLELLA, G., SEBASTIO, G. and BARALLE, F.E., Transformed human cells produce a new fibronectin isoform by preferential alternative splicing of a previously unobserved exon. *EMBO J.*, **6**, 2337–2342 (1987).

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Thorpe et al., "Selective Killing of Proliferating Vascular Endothelial Cells by an Anti-Fibronectin Receptor Immunotoxin," 16th LH Gray Conference, University of Manchester Institute of Science and Technology, Sep. 17-21, 1990.

Other Reference Publication (89):
Aoyagi, "Distribution of Plasma Fibronectin in the Metastatic Lesion of Cancer: Experimental Study by Autoradiography," Thrombosis Research, 49:265-75, 1988.

Other Reference Publication (96):
Burton-Wurster, et al., "Expression of the Ed B Fibronectin Isoform in Adult Human Articular Cartilage," Biochemical and Biophysical Research Communication 165(2): 782-87, 1989.

Other Reference Publication (98):
Carnemolla et al., "A Tumor-associated Fibronectin Isoform Generated by Alternative Splicing of Messenger RNA Precursors," The Journal of Cell Biology, 108:1139-48, 1989.

Other Reference Publication (99):
Carnemolla et al., "The Inclusion of the Type III Repeat ED-B in the Fibronectin Molecule Generates Conformational Modifications that Unmask a Cryptic Sequence," The Journal of Biological Chemistry, 267(34):245898-92, 1992.

Other Reference Publication (100):
Carnemolla, et al., "Phage Antibodies with Pan-Species Recognition of the Oncofoetal Angiogenesis Marker Fibronectin ED-B Domain," Int. J. Cancer 68:397-505, 1996.

Other Reference Publication (101):
Castellani, et al., "The Fibronectin Isoform Containing the ED-B Oncofoetal Domain: A Marker of Angiogenesis," Int. J. Cancer, 59:612-18, 1994.

Other Reference Publication (104):
Farnoud, et al., "Fibronectin Isoforms Are Differentially Expressed in Normal and Adenomatous Human Anterior Pituitaries," Int. J. Cancer, 61:27-34, 1995.

Other Reference Publication (108):
Kaczmarek, et al., "Distribution of Oncofetal Fibronectin Isoforms in Normal Hyperplastic and Neoplastic Human Breast Tissues," Int. J. Cancer, 58:11-16, 1994.

Other Reference Publication (112):
Linnala, et al., "Human Amnion Epithelial Cells Assemble Tenascins and Three Fibronectin Isoforms in the Extracellular Matrix," FEBS, 314(1,2):74-78, 1993.

Other Reference Publication (115):
Oyama, et al., "Coordinate Oncodevelopmental Modulation of Alternative Splicing of Fibronectin Pre-Messenger RNA at ED-A, ED-B and CS1 Regions in Human Liver Tumors," Cancer Research 53:2005-11, 1993.

Other Reference Publication (116):
Peters, et al., "Expression of the Alternatively Spliced EIIB Segment of Fibronectin," Cell Adhesion and Communication, 3:67-89, 1995.

Other Reference Publication (126):
Saiki et al., "Inhibition of Tumor Angiogenesis by a Synthetic Cell-Adhesive Polypeptide Containing the Arg-Gly-Asp (RGD) Sequence of Fibronectin, Poly(RGD)," Jpn. J. Cancer Res. 81:668-75, 1990.

Other Reference Publication (128):
Schwarzbauer, "Alternative Splicing of Fibronectin: Three Variants, Three Functions," BioEssays 13(10)527-33, 1991.

Other Reference Publication (133):
Ueda, et al., "Selective Distribution of Fibronectin to a Tumor-Cell Line," Cancer Letters 31:261-65, 1986.

Other Reference Publication (134):
Vartio, et al, "Differential Expression of the ED Sequence-Containing Form of Cellular Fibronectin in Embryonic and Adult Human Tissues," Journal of Cell Science, 88:419-30, 1987.

Jpn. J. Cancer Res. 81, 668-675, June/July 1990

Inhibition of Tumor Angiogenesis by a Synthetic Cell-adhesive Polypeptide Containing the Arg-Gly-Asp (RGD) Sequence of Fibronectin, Poly(RGD)

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We have investigated the anti-angiogenic effect of a polymeric peptide based on the Arg-Gly-Asp (RGD) core sequence of fibronectin as a monomer unit, i.e., poly(RGD), in syngeneic mice and *in vitro*. Single intratumoral administration of poly(RGD) on day 0, 1 or 7 after tumor implantation achieved a significant reduction of B16-BL6 melanoma colonization in the lungs, but did not affect the size of the primary tumor at the time of amputation. The number of capillary blood vessels oriented toward the tumor mass increased during the early growth phase after the intradermal inoculation of the tumor. Poly(RGD) significantly inhibited the formation of tumor neovascularization when co-injected with the tumor cells or separately injected intratumorally or intravenously on day 1 or 3 after tumor inoculation. This inhibitory effect of poly(RGD) was dose-dependent. Poly(RGD) was able to inhibit the haptotactic migration of endothelial cells along a gradient of substratum-immobilized fibronectin but not laminin. Tumor-conditioned medium (CM) by itself did not act as a chemoattractant when it was added in the lower compartment of a Transwell chamber, but promoted the endothelial cell migration to immobilized fibronectin or laminin. Poly(RGD) inhibited the enhanced cell migration to fibronectin but not to laminin in response to CM. Thus, poly(RGD)-mediated inhibition of tumor metastasis may be partly due to the inhibition of tumor-induced angiogenesis at primary and secondary sites.

Key words: RGD peptide —Tumor angiogenesis — Endothelial cell migration — Metastasis

Angiogenesis, the growth of new capillary blood vessels in the host, is a characteristic phenomenon common to most solid malignant tumors. Following establishment of an adequate blood supply, tumor cells will not only grow but also acquire metastatic potential to distant tissues or organs.[1] Tumor angiogenesis is induced by angiogenic factors produced by tumor cells (called tumor angiogenesis factor) which activate and attract endothelial cells.[2-4] This response of microvascular endothelial cells to such soluble factors consists of three major components; increased rate of cell proliferation, a stimulation of endothelial cell migration along a gradient of angiogenic factor and an increased production of proteolytic enzymes.[5] Also angiogenesis is not usually active in the normal adult except during wound repair, ovulation and menstruation. Therefore the inhibition of angiogenesis (neovascularization) may provide a means to control tumor growth and metastasis.[3,6]

Tumor angiogenesis was found to be inhibited by several natural products such as protamine,[7] a purified cartilage extract[8] and medroxyprogesterone.[9] Folkman *et al.*[10] reported that a combination of cortisone acetate and heparin inhibited growth of new blood vessels toward the tumor mass and resulted in regression of several experimental tumors in mice. Eisenstein *et al.*[11] showed that cartilage extract inhibited the growth of aortic endothelial cells in culture. We have reported that a polypeptide based on the Arg-Gly-Asp (RGD) sequence of fibronectin as a monomer unit, poly(RGD), could dramatically inhibit experimental and spontaneous lung metastases of B16-BL6 melanoma cells possibly through an inhibitory action at some points in the adhesion, migration and invasion of tumor cells during the metastatic process.[12,13] Poly(RGD) was also found to interfere with the interaction between tumor cells and platelets including tumor-elicited platelet aggregation, which may enhance the survival, arrest and invasiveness of tumors.[14]

We describe here the effect of RGD-containing polypeptides on tumor angiogenesis caused by tumor inoculation in syngeneic mice. We also examine the influence of polypeptides on endothelial cell migration in response to immobilized adhesive proteins and/or the conditioned medium of tumor cells *in vitro*.

MATERIALS AND METHODS

Cells and cell culture Highly metastatic B16-BL6 melanoma cells, obtained by an *in vitro* selection procedure for invasion,[15] were kindly provided by Dr. I. J. Fidler,

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M.D. Anderson Cancer Center, Houston, TX. Melanoma cells were maintained as monolayer cultures in Eagle's minimal essential medium (MEM) supplemented with 7.5% fetal bovine serum (FBS), vitamin solution, sodium pyruvate, nonessential amino acids and L-glutamine. Rat lung endothelial cells (RLE cells) were kindly provided by Dr. M. Nakajima, M.D. Anderson Cancer Center, Houston, TX. RLE cells were maintained as monolayer cultures in 1% gelatin-coated culture dishes containing a 1:1 ratio of DMEM and Ham's nutrient mixture F12 medium (DMEM:F12; GIBCO Laboratories, Logan, UT) and 100 μg/ml of endothelial mitogen (Biomedical Technologies, Inc., Cambridge, MA).

Synthetic polymeric peptides and reagents The polypeptide based on the Arg-Gly-Asp (RGD) sequence of fibronectin, poly(RGD), was prepared by synthesis of the monomer peptide, RGD, by a conventional method, followed by polymerization with diphenylphosphoryl azide, as described elsewhere.[12,16] Poly(RGD) consists of a sequence of RGD units, whereas poly(R,G,D) is a polymer with randomly arranged sequences of the three amino acids. Hence, in the sequence of poly(RGD), the G residue always lies between R and D residues, and the -RGD- sequence is present as a segment. In the sequence of poly(R,G,D), on the other hand, the three amino acids are randomly arranged and the probability of their forming the -RGD- sequence is statistically very small. The polymeric peptides used are estimated to have an average molecular weight of approximately 10,000, as assessed by SDS-polyacrylamide gel electrophoresis and gel permeation chromatography. These polypeptides and Arg-Gly-Asp-Ser (RGDS) monomer peptide were each dissolved in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) before use. Purified mouse fibronectin was purchased from Seikagaku Kogyo Co. Ltd., Tokyo. Purified laminin was obtained from Collaborative Research Inc., MA. Clupein (protamine from herring testis) was purchased from Sigma Chemical Co., MO. All the reagents and media in this study were endotoxin-free (approximately < 1.0 ng/ml) as determined by a colorimetric assay (Pyrodick, Seikagaku Kogyo Co. Ltd.).

Spontaneous lung metastasis Mice were given subcutaneous (s.c.) injections of B16-BL6 melanoma cells (5×10^5) into the right hind footpad. Polymeric peptides were administered intratumorally (i.t.) on various days after tumor inoculation, and then primary tumors were surgically removed by amputation on day 21. Mice were killed 14 days after the amputation. The lungs were fixed in Bouin's solution and lung tumor colonies were counted under a dissecting microscope.

Tumor angiogenesis in mice The assay for tumor angiogenesis in syngeneic mice was carried out according to the method described by Kreisle and Ershler[17] with some modifications. C57BL/6 mice were injected intradermally (i.d.) with 5×10^5 B16-BL6 melanoma cells (though a 27-gauge needle) at two sites on the back. Polypeptides were administered i.t. or intravenously (i.v.) at various days after tumor inoculation. At 72 h after the administration of polypeptide, the mice were killed immediately after i.v. injection (0.2 ml) of 0.1% Evans blue and the skin was separated from the underlying tissue. Angiogenesis was quantitated by counting the number of vessels oriented towards the tumor mass (as shown in Fig. 1) under a dissecting microscope. The size of the cell mass was measured by averaging the long and short diameters. All counts were made by a single observer in a blind manner.

Cell migration assay Endothelial cell migration along a gradient of substratum-bound fibronectin or laminin (haptotaxis) in response to tumor conditioned medium was assayed in Transwell cell culture chambers (Costar No. 3422, Cambridge, MA) as described previously.[18] Briefly, polyvinylpyrrolidone-free polycarbonate filters with 8 μm pore size (Nucleopore Corp., Pleasanton, CA) were precoated with 5 μg of fibronectin or laminin in a volume of 50 μl on the lower surface, and dried overnight at room temperature. The coated filters were washed extensively in PBS and then dried immediately before use. Log-phase cell cultures of RLE cells were harvested with 1 m*M* EDTA in PBS, washed three times with serum-free MEM, and resuspended to a final concentration of 2×10^6/ml in MEM containing 0.1% bovine serum albumin (BSA). Cell suspensions (100 μl) with or without polypeptides were added to the upper compartment, and incubated for 4 h at 37°C in the absence or presence of B16-BL6 cells themselves or their conditioned medium (CM), obtained after a 24-h incubation of tumor cells in serum-free MEM, in the lower compartment. The filters were fixed with methanol, and stained with hematoxylin and eosin. The cells on the upper surface of filters were removed by wiping with a cotton swab. The cells that had migrated to various areas of the lower surface were manually counted under a microscope at a magnification of 400, and each assay was performed in triplicate.

Statistical analysis The significance of the differences between groups was calculated by applying Student's two-tailed *t* test.

RESULTS

Inhibition of spontaneous lung metastasis by polypeptides We first examined the effect of our original polymeric peptides containing the RGD sequence on the spontaneous lung metastasis of metastatic B16-BL6 melanoma (Table I). Polypeptides were administered i.t. into the right hind footpad with an advanced primary

Table I. Effect of Poly(RGD) on Spontaneous Lung Metastasis Caused by Intrafootpad Injection of B16-BL6 Melanoma Cells

Administered i.t. with		Timing	Primary tumor size on day 21 (mm · SD)	No. of lung metastases mean · SD (range)	$P^{a)}$
Expt. I					
Untreated (PBS)			9 · 2	44 · 22 (22-80)	
Poly(RGD)	100 μg	on day 0	7 · 3	6 · 6 (0-14)	0.001
		day 1	8 · 2	6 · 6 (0-14)	0.001
		day 7	8 · 2	6 · 4 (0-10)	0.001
		day 14	9 · 2	42 · 26 (14-86)	
Expt. II					
Untreated (PBS)			10 · 3	46 · 4 (43-51)	
Poly(RGD)	100 μg	on day 7	10 · 3	12 · 6 (9-21)	0.001
Poly(R,G,D)	100 μg	on day 7	11 · 3	58 · 23 (43-84)	

Five C57BL/6 mice per group were administered i.t. with 100 μg of polypeptides at the indicated times after tumor inoculation. Primary tumors were surgically removed on day 21 and mice were killed 2 weeks after the amputation.

a) Compared with untreated control by Student's two-tailed *t* test.

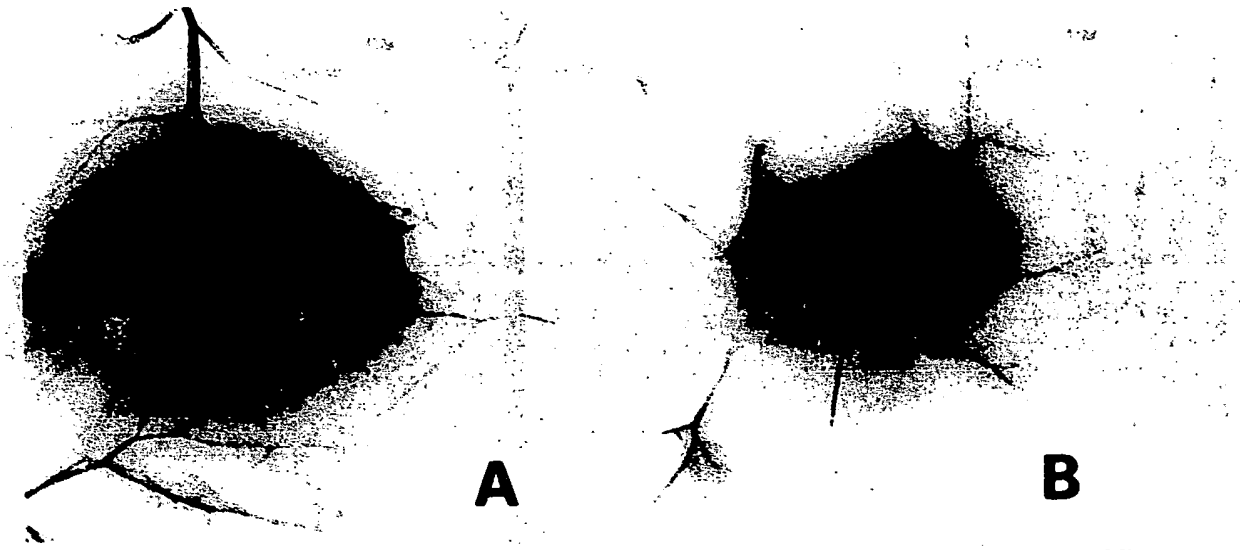

Fig. 1. Photomicrographs of angiogenesis induced at 3 days by B16-BL6 melanoma implanted into C57BL/6 mice. Mice were injected i.d. on the back with B16-BL6 melanoma cells (5×10^5) admixed with (B) or without (A) 100 μg of poly(RGD). Three days after tumor inoculation, mice were killed immediately after i.v. injection (0.2 ml) of 0.1% Evans blue and the skin was separated from the underlying tissues.

tumor at various times following tumor inoculation, after which, n day 21, the primary tumors were surgically removed. Tumor colonies in the lung were monitored 14 days after tumor excision. Single i.t. administration of poly(RGD) on day 0 (mixture), day 1 or day 7 caused a marked reduction of tumor colonies f B16-BL6 melanoma, but did not affect the growth (size) of primary tumors at the time of amputation on day 21 compared with the untreated control. In contrast, the administration of the random polypeptide, poly(R,G,D), on day 7 after tumor inoculation failed to inhibit the lung metastases. These results are in accordance with our

previous reports on the inhibition of spontaneous lung metastasis by i.t. or i.v. administrations of polypeptides.[12,13] We also observed that the polypeptide had no direct cytotoxic effect on such cells as the B16-BL6 cells, endothelial cells, mouse red blood cells or thymocytes *in vitro*, nor did it affect their cell growth or the aggregation state of serum proteins (data not shown).

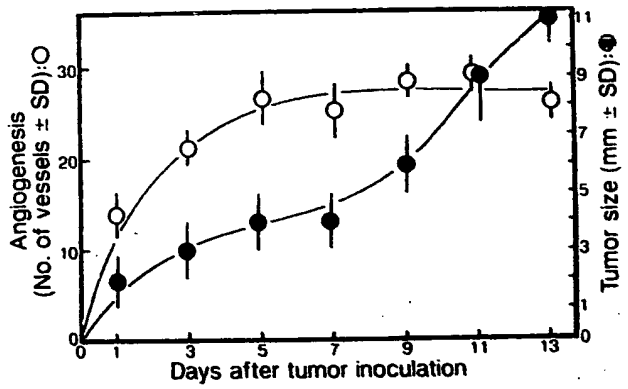

Fig. 2. Time course of angiogenesis and tumor growth at the injection sites. Three C57BL/6 mice per group were injected i.d. with B16-BL6 melanoma cells (5×10^5) at two sites on the back. At various times after tumor inoculation, mice were killed and the skin was separated from the underlying tissues.

Inhibition of *in vivo* tumor angiogenesis by poly(RGD) Progressive tumor growth and metastases appear to be associated with the growth of persistent capillary blood vessels (neovascularization) at the primary and metastatic sites.[3,4] We therefore tested the time-course of angiogenesis and growth of B16-BL6 cells at the injection site according to the method described previously.[17] At various times after tumor inoculation on the back of mice, the number of vessels oriented towards the tumor mass on the separated skins (angiogenesis) was measured as a function of time under a dissecting microscope (Fig. 1). Figure 2 shows the relationship between angiogenic response and size of tumor cell mass following i.d. inoculation of B16-BL6 melanoma cells. The size (growth) of tumor cells after the inoculation increased in a time-dependent manner. In contrast, angiogenic response increased logarithmically over 5 days, and thereafter little or no increase in the number of vessels was observed. It appears that the vessels became dilated with the growth of the tumor mass and thereby increased the blood supply. However, no leakage of dye (Evans blue) into the surrounding region was observed after the i. v. injection. The results indicate that the formation of capillary blood vessels increasingly occurred during the early growth phase after tumor inoculation. We next examined the effect of antimetastatic poly(RGD) on tumor angiogenesis caused by i.d. tumor inoculation into syngeneic mice. Mice were treated with 100 μg of poly(RGD) on

Table II. Inhibition of Tumor Angiogenesis by Polypeptides

Administered with	Route	Angiogenesis (No. of vessels ± SD)	Tumor size (mm ± SD)
Day 0 (co-injection)			
Control (PBS)	i.d.	21 ± 3	3 ± 1
Poly(RGD)	i.d.	12 ± 2**	3 ± 1
RGDS	i.d.	21 ± 5	3 ± 1
Clupein	i.d.	14 ± 2**	3 ± 1
Day 1			
Control (PBS)	i.t.	24 ± 2	3 ± 1
Poly(RGD)	i.t.	15 ± 3**	3 ± 1
	i.v.	19 ± 2*	3 ± 1
RGDS	i.t.	21 ± 3	3 ± 1
Day 3			
Control	i.t.	27 ± 1	4 ± 1
Poly(RGD)	i.t.	22 ± 2*	4 ± 1
RGDS	i.t.	28 ± 2	4 ± 1

Three C57BL/6 mice per group were treated with 100 μg of polypeptides on day 0, 1 or 3 after i.d. injection of B16-BL6 melanoma cells (5×10^5) at two sites on the back. Three days after the administration, mice were killed immediately after i.v. injection (0.2 ml) of 0.1% Evans blue and the skin was separated from the underlying tissue. Angiogenesis was quantitated by counting the number of vessels oriented towards the tumor mass.
**, $P < 0.001$; *, $P < 0.01$.

Table III. Dose-Response Relation for Anti-angiogenic Activity of Poly(RGD) *in vivo*

Administered i.t. with	Dose (μg/mouse)	Angiogenesis (No. of vessels ± SD)	Tumor size (mm ± SD)
Control (PBS)		20 ± 2	3 ± 1
Poly(RGD)	20	20 ± 1	4 ± 2
	100	12 ± 1*	3 ± 1
	500	8 ± 2*	4 ± 1
Clupein	100	8 ± 1*	3 ± 1

Three C57BL/6 mice per group were administered i.t. with various doses of poly(RGD) on day 1 after i.d. inoculation of B16-BL6 melanoma cells (5×10^5) at two sites on the back. Three days after the administration, mice were killed immediately after i.v. injection (0.2 ml) of 0.1% Evans blue and the skin was separated from the underlying tissue. Angiogenesis was quantitated by counting the number of vessels oriented toward the tumor mass.
*, $P < 0.001$.

Table IV. Effect of Polypeptides on the Haptotactic Migration of Endothelial Cells to Fibronectin- or Laminin-coated Filters

Filters precoated (on the lower surface) with		Added in the upper compartment		No. of migrated cells (mean ± SD)	$P^{a)}$
BSA	5 μg			1 ± 1	
Fibronectin	0.005			24 ± 3	
	0.05			40 ± 3	
	0.5			70 ± 7	
Fibronectin	5	—		74 ± 10	
		poly(RGD)	20 μg/ml	67 ± 4	
			100	46 ± 3	0.001
			500	38 ± 4	0.001
		RGDS	500	62 ± 5	
		poly(R,G,D)	500	60 ± 7	
Laminin	5	—		34 ± 3	
		poly(RGD)	500 μg/ml	33 ± 5	

Endothelial cells (RLE cells, 2×10^5) were seeded on filters precoated on the lower surface with fibronectin or laminin. Polypeptides were added to the upper compartments of Transwell chambers. After a 4-h incubation, the migrant cells on the lower surface of the filters were counted visually.
a) Compared with the untreated control by Student's two-tailed *t* test.

various days after i.d. injection of B16-BL6 cells, and angiogenic activity was assessed 3 days after the administration of the polypeptide. Table II shows that poly(RGD) significantly inhibited the angiogenic response when it was co-injected i.d. with tumor cells, or separately injected i.t. or i.v. on day 1 or 3 after tumor implantation. RGDS monomer peptide at the dose of 100 μg did not affect the number of vessels as compared with the untreated control. Taylor and Folkman[7] have shown that protamine is a specific inhibitor of angiogenesis. Clupein, a protamine derived from herring testis, was also able to inhibit tumor angiogenesis. However, anti-angiogenic poly(RGD) as well as clupein did not show any inhibitory effect on the tumor size (growth) at the injection sites. As shown in Table III, poly(RGD) inhibited tumor neovascularization in a dose-dependent manner upon i.t. administration on day 1 after tumor inoculation.

Effect of poly(RGD) on haptotactic migration of endothelial cells Since an essential component of tumor neovascularization is the migration of vascular endothelial cells from existing blood vessels toward tumor masses,[19,20] we examined the effect of poly(RGD) on the haptotactic migration of endothelial cells (RLE cells) to fibronectin- or laminin-coated filters. Table IV shows that RLE cells migrated to the lower surface of the

Table V. Effect of Poly(RGD) on Endothelial Cell Migration on Fibronectin- or Laminin-coated Filters in Response to Tumor Cell Conditioned Medium

Filters precoated (on the lower surface) with	Added to upper compartment	Added to lower compartment	No. of migrated cells (mean ± SD)	P
BSA			1 ± 1	
		CM	0 ± 0	
Fibronectin, 5 μg			38 ± 2	
		CM	70 ± 6	
	poly(RGD), 100 μg/ml	CM	47 ± 4	< 0.005
	500	CM	36 ± 5	< 0.001
	RLE cells pretreated with poly(RGD),[a] 500 μg/ml	CM	29 ± 6	< 0.001
Laminin, 5 μg			35 ± 5	
		CM	77 ± 8	
		CM + poly(RGD), 500 μg/ml	71 ± 8	
	poly(RGD), 500 μg/ml	CM	73 ± 8	
	poly(RGD), 500 μg/ml	CM + poly(RGD), 500 μg/ml	75 ± 5	
		Endothelial mitogen, 100 μg/ml	133 ± 14	

Endothelial cells (RLE cells, 2×10^5) were seeded on filters precoated on the lower surface with fibronectin or laminin, and incubated with conditioned medium (CM) in the lower compartment. Poly(RGD) was added to the upper or lower compartment of the Transwell chamber. After a 4-h incubation, the migrant cells on the lower surface were counted visually.

a) RLE cells were pretreated with 500 μg/ml poly(RGD) at 37 C for 1 h.

fibronectin-coated filters in a dose-dependent manner, but did not migrate to BSA-coated filters. Poly(RGD) inhibited haptotactic migration of RLE cells to fibronectin-coated filters in a dose-dependent fashion when it was added to the upper compartment of the chamber. In contrast, RGDS or random polypeptide, poly(R,G,D), did not show any inhibition even at the concentration of 500 μg/ml. On the other hand, RLE cells were able to migrate to laminin-coated filters, but poly(RGD) did not affect this migration. We also observed that fibronectin promoted such haptotactic migration of RLE cells to laminin-coated filters in a concentration-dependent manner when it was added freely in the lower compartment (data not shown). We next investigated the influence of poly(RGD) and/or tumor CM on haptotactic migration of RLE cells to fibronectin- or laminin-immobilized filters *in vitro* (Table V). CM was able to promote haptotactic migration of RLE cells to fibronectin- or laminin-coated filters when it was added to the lower compartment of the chamber, whereas it did not affect the cell migration to BSA-coated filters. These results imply that CM itself was unable to act as a chemoattractant but was able to promote haptotaxis of RLE cells on immobilized fibronectin or laminin substrates. The addition of poly(RGD) to the upper compartment or the pretreatment of RLE cells with poly(RGD) inhibited the enhanced haptotaxis of RLE cells to fibronectin-coated filters in response to CM. In contrast, poly(RGD) did not inhibit the haptotactic migration to laminin-coated filters in response to CM when it was added freely to the upper or lower compartment, or both compartments. This result indicates that poly(RGD) was specifically able to inhibit the cell migration to fibronectin substrates (but not to laminin substrates) in the absence or presence of CM. Also the addition of B16-BL6 cells themselves or endothelial mitogen, in place of CM, to the lower compartment promoted the migration of RLE cells to fibronectin substrate.

DISCUSSION

Progressive tumor growth followed by metastasis appears to be associated with the creation of extensive vascular networks (neovascularization) at primary and/or secondary tumor sites.[3,4] We here focused our attention on the tumor-induced angiogenesis, and examined the effect of our polymeric peptide, poly(RGD), on the induction of neovascularization caused by the inoculation of tumor cells in syngeneic mice and on the migration of endothelial cells in response to tumor-conditioned medium *in vitro*, in order to elucidate the cellular basis of neovascularization. Poly(RGD) significantly reduced the number of capillary vessels oriented towards the tumor mass when it was co-injected i.d. with tumor cells

or separately injected i.t. or i.v. on day 1 or 3 after tumor inoculation in syngeneic mice (Tables II and III, and Fig. 1). However, RGDS monomer peptide did not show any effect at the dose of 100 μg. Since poly(RGD) had no toxic effect on tumor cells *in vitro* and no short-term toxicity to the host, the inhibitory effect on tumor neovascularization may not depend on the direct cytotoxicity.

In the spontaneous metastasis model, a single i.t. administration of non-toxic poly(RGD) at an early stage (day 0, 1 or 7) after tumor inoculation caused a significant reduction of tumor colonies in the lung, but did not affect the growth of the primary tumors (Table I). The random polypeptide, poly(R,G,D), in which the three amino acids (R, G and D) were randomly arranged, and the RGDS monomer peptide showed no antimetastatic activity even on i.t. administration. This result indicates that the inhibitory effect of poly(RGD) on lung metastasis by i.t. administration may be partly due to the inhibition of angiogenic response at an early phase after tumor inoculation, resulting in interference with active migration or release of tumor cells from the primary tumor. We have also reported that multiple i.v. administrations of poly(RGD) before or after surgical excision of the primary tumor resulted in a significant reduction of lung tumor colonies, possibly through some inhibitory action on the adhesion, migration and invasion of tumor cells or on tumor-induced platelet aggregation.[12-14] However, since angiogenesis is necessary for the growth of pulmonary metastatic tumors as well as for the growth of primary tumors, the i.v. administration of poly(RGD) may affect the angiogenic response at pulmonary metastatic sites as well as at the primary tumor site (Table II). Thus, the inhibition of tumor angiogenesis by i.t. or i.v. administration of poly(RGD) may in part reflect the antimetastatic effect. We are currently characterizing the kinetics of the antiangiogenic effect of poly(RGD) and seeking the most effective treatment modalities, including timings or schedule of administration, etc.

Endothelial cell migration toward tumor masses following the formation of capillary sprouts is an essential component in the process of tumor neovascularization.[19,20] To analyze further the antiangiogenic effect of poly(RGD), we investigated the effect of poly(RGD) on haptotactic migration of endothelial cells (RLE cells) in response to a gradient of substratum (filter)-immobilized extracellular matrix proteins such as fibronectin or laminin (Table IV). RLE cells migrated to the lower surface of the filter bearing either fibronectin or laminin, but not BSA, thus indicating that fibronectin or laminin can serve as a signal for endothelial migration. The addition of poly(RGD) in the upper compartment of the chamber resulted in the inhibition of haptotactic migration to the fibronectin-coated filter but not to the laminin-coated filter.

Factors secreted by the tumor itself (e.g. tumor angiogenic factor) have been shown to promote angiogenesis (neovascularization) at the site of the tumor.[2-4] Zetter[21] reported that the random locomotion of capillary endothelial cells (chemokinesis) increased when they were cultured with tumor-conditioned medium. Several investigators have reported that aortic endothelial cells responded chemotactically to crude homogenates of bovine retina, endothelial mitogen, soluble fibronectin or tumor angiogenesis factor from Walker 256 tumor cells.[22-24] As shown in Table V, the addition of B16-BL6 cell CM to the lower compartment of the Transwell chamber did not cause any migration of endothelial cells to BSA-coated substrate, but augmented haptotaxis to fibronectin- or laminin-immobilized substrates. We also observed that, in place of CM, fibroblast growth factor (FGF), epidermal growth factor (EGF) or transforming growth factor-α (TGF-α), which are known to induce angiogenesis, enhanced the haptotactic migration of RLE cells to fibronectin substrate (data not shown). Therefore further study will be needed to determine whether or not the active substance in CM is identical with such known growth factors. The addition of poly(RGD) in the upper compartment or pretreatment of RLE cells with poly(RGD) resulted in the inhibition of both haptotactic migration to fibronectin substrate and the enhanced haptotactic migration to fibronectin-substrate in response to CM. In contrast, poly(RGD) did not affect the haptotactic migration to laminin substrate or the enhanced migration to laminin substrate in response to CM. This result indicates that poly(RGD)-mediated inhibition of tumor neovascularization is attributable to the inhibition of endothelial migration along a gradient of extracellular matrix fibronectin in conjunction with CM, and is substrate-specific. In addition, poly(RGD) did not directly affect the migratory effect of CM. The inhibitory mechanism by poly(RGD) may therefore depend on interference with the interaction between endothelial cells (probably the surface receptor) and fibronectin. Further analysis will be needed to establish the mechanism.

In conclusion, we have demonstrated that non-toxic, antimetastatic poly(RGD) inhibited both tumor angiogenesis caused by tumor inoculation in syngeneic mice and endothelial cell migration to immobilized fibronectin in the presence or absence of tumor-conditioned medium *in vitro*. The mechanism responsible for the inhibition of spontaneous lung metastasis by our synthetic polymeric peptide may be more complex than a simple inhibition of tumor angiogenesis. Thus, the use of poly(RGD) might offer a promising therapeutic basis for preventing cancer metastasis.

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REFERENCES

1) Schor, A. M. and Schor, S. L. Tumor angiogenesis. *J. Pathol.*, **141**, 385–413 (1983).
2) Folkman, J., Merler, E., Abernathy, C. and Williams, G. Isolation of a tumor factor responsible for angiogenesis. *J. Exp. Med.*, **133**, 275–288 (1971).
3) Folkman, J. Tumor angiogenesis. *Adv. Cancer Res.*, **43**, 175–203 (1985).
4) Folkman, J. and Klagsbrun, M. Angiogenic factors. *Science*, **235**, 442–447 (1987).
5) Presta, M., Moscatelli, D., Joseph-Silverstein, J. and Rifkin, D. B. Purification from a human hepatoma cell line of a basic fibroblast growth factor-like molecule that stimulates capillary endothelial cell plasminogen activator production, DNA synthesis, and migration. *Mol. Cell. Biol.*, **6**, 4060–4066 (1986).
6) Folkman, J. Tumor angiogenesis: therapeutic implications. *N. Engl. J. Med.*, **18**, 1182–1186 (1971).
7) Taylor, S. and Folkman, J. Protamine is an inhibitor of angiogenesis. *Nature*, **297**, 307–312 (1982).
8) Lee, A. and Langer, R. Shark cartilage contains inhibitors of tumor angiogenesis. *Science*, **221**, 1185–1187 (1983).
9) Gross, J., Azizkhan, R. G., Biswas, C., Bruns, R. R., Hsieh, D. S. and Folkman, J. Inhibition of tumor growth, vascularization and collagenolysis in the rabbit cornea by medroxyprogesterone. *Proc. Natl. Acad. Sci. USA*, **78**, 1176–1180 (1981).
10) Folkman, J., Langer, R., Linhardt, R. J., Haudenschild, C. and Taylor, S. Angiogenesis inhibition and tumor regression caused by heparin or a heparin fragment in the presence of cortisone. *Science*, **221**, 719–725 (1983).
11) Eisenstein, R., Kuettner, K. E., Soble, L. W. and Sorgente, N. The resistance of certain tissues to invasion: penetrability of explanted tissues by vascularized mesenchyme. *Am. J. Pathol.*, **73**, 765–774 (1973).
12) Saiki, I., Iida, J., Murata, J., Ogawa, R., Nishi, N., Sugimura, K., Tokura, S. and Azuma, I. Inhibition of the metastasis of murine malignant melanoma by synthetic polymeric peptides containing core sequence of cell-adhesive molecules. *Cancer Res.*, **49**, 3815–3822 (1989).
13) Saiki, I., Murata, J., Iida, J., Nishi, N., Sugimura, K. and Azuma, I. The inhibition of murine lung metastasis by synthetic polypeptides [poly(arg-gly-asp) and poly(tyr-ile-gly-ser-arg)] with a core sequence of cell adhesion molecules. *Br. J. Cancer*, **59**, 194–197 (1989).
14) Saiki, I., Murata, J., Iida, J., Sakurai, T., Nishi, N., Matsuno, K. and Azuma, I. Antimetastatic effects of synthetic polypeptides containing repeated structures of the cell adhesive Arg-Gly-Asp (RGD) and Tyr-Ile-Gly-Ser-Arg (YIGSR) sequences. *Br. J. Cancer*, **60**, 722–728 (1989).
15) Hart, I. R. The selection and characterization of an invasive variant of B16 melanoma. *Am. J. Pathol.*, **97**, 587–592 (1979).
16) Murata, J., Saiki, I., Iida, J., Azuma, I., Kawahara, H., Nishi, N. and Tokura, S. Inhibition of tumor cell adhesion by anti-metastatic polypeptide containing a repetitive Arg-Gly-Asp sequence. *Int. J. Biol. Macromol.*, **11**, 226–232 (1989).
17) Kreisle, R. A. and Ershler, W. B. Investigation of tumor angiogenesis in an id mouse model: role of host-tumor interactions. *J. Natl. Cancer Inst.*, **80**, 849–854 (1988).
18) McCarthy, J. B., Palm, S. L. and Furcht, L. T. Migration by haptotaxis of a Schwann cell tumor line to the basement membrane glycoprotein laminin. *J. Cell Biol.*, **97**, 772–777 (1983).
19) Ausprunk, D. H. and Folkman, J. Migration and proliferation of endothelial cells in preformed and newly formed blood vessels during tumor angiogenesis. *Microvasc. Res.*, **14**, 53–65 (1977).
20) Bowersox, J. C. and Sorgente, N. Chemotaxis of aortic endothelial cells in response to fibronectin. *Cancer Res.*, **42**, 2547–2551 (1982).
21) Zetter, B. R. Migration of capillary endothelial cells is stimulated by tumor-derived factors. *Nature*, **285**, 41–43 (1980).
22) Glaser, B. M. and D'Amore, P. A. Adult tissues contain chemoattractants for vascular endothelial cells. *Nature*, **288**, 483–484 (1980).
23) McAuslan, B. R. and Hoffman, H. Endothelium stimulating factor from Walker carcinoma cells. *Exp. Cell Res.*, **119**, 181–190 (1979).
24) Phillips, P., Steward, J. K. and Kumar, S. Tumor angiogenesis factor (TAF) in human and animal tumors. *Int. J. Cancer*, **17**, 549–558 (1976).

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Thorpe et al., "Selective Killing of Proliferating Vascular Endothelial Cells by an Anti-Fibronectin Receptor Immunotoxin," 16th LH Gray Conference, University of Manchester Institute of Science and Technology, Sep. 17-21, 1990.

Other Reference Publication (89):
Aoyagi, "Distribution of Plasma Fibronectin in the Metastatic Lesion of Cancer: Experimental Study by Autoradiography," Thrombosis Research, 49:265-75, 1988.

Other Reference Publication (96):
Burton-Wurster, et al., "Expression of the Ed B Fibronectin Isoform in Adult Human Articular Cartilage," Biochemical and Biophysical Research Communication 165(2): 782-87, 1989.

Other Reference Publication (98):
Carnemolla et al., "A Tumor-associated Fibronectin Isoform Generated by Alternative Splicing of Messenger RNA Precursors," The Journal of Cell Biology, 108:1139-48, 1989.

Other Reference Publication (99):
Carnemolla et al., "The Inclusion of the Type III Repeat ED-B in the Fibronectin Molecule Generates Conformational Modifications that Unmask a Cryptic Sequence," The Journal of Biological Chemistry, 267(34):245898-92, 1992.

Other Reference Publication (100):
Carnemolla, et al., "Phage Antibodies with Pan-Species Recognition of the Oncofoetal Angiogenesis Marker Fibronectin ED-B Domain," Int. J. Cancer 68:397-505, 1996.

Other Reference Publication (101):
Castellani, et al., "The Fibronectin Isoform Containing the ED-B Oncofoetal Domain: A Marker of Angiogenesis," Int. J. Cancer, 59:612-18, 1994.

Other Reference Publication (104):
Farnoud, et al., "Fibronectin Isoforms Are Differentially Expressed in Normal and Adenomatous Human Anterior Pituitaries," Int. J. Cancer, 61:27-34, 1995.

Other Reference Publication (108):
Kaczmarek, et al., "Distribution of Oncofetal Fibronectin Isoforms in Normal Hyperplastic and Neoplastic Human Breast Tissues," Int. J. Cancer, 58:11-16, 1994.

Other Reference Publication (112):
Linnala, et al., "Human Amnion Epithelial Cells Assemble Tenascins and Three Fibronectin Isoforms in the Extracellular Matrix," FEBS, 314(1,2):74-78, 1993.

Other Reference Publication (115):
Oyama, et al., "Coordinate Oncodevelopmental Modulation of Alternative Splicing of Fibronectin Pre-Messenger RNA at ED-A, ED-B and CS1 Regions in Human Liver Tumors," Cancer Research 53:2005-11, 1993.

Other Reference Publication (116):
Peters, et al., "Expression of the Alternatively Spliced EIIB Segment of Fibronectin," Cell Adhesion and Communication, 3:67-89, 1995.

Other Reference Publication (126):
Saiki et al., "Inhibition of Tumor Angiogenesis by a Synthetic Cell-Adhesive Polypeptide Containing the Arg-Gly-Asp (RGD) Sequence of Fibronectin, Poly(RGD)," Jpn. J. Cancer Res. 81:668-75, 1990.

Other Reference Publication (128):
Schwarzbauer, "Alternative Splicing of Fibronectin: Three Variants, Three Functions," BioEssays 13(10)527-33, 1991.

Other Reference Publication (133):
Ueda, et al., "Selective Distribution of Fibronectin to a Tumor-Cell Line," Cancer Letters 31:261-65, 1986.

Other Reference Publication (134):
Vartio, et al, "Differential Expression of the ED Sequence-Containing Form of Cellular Fibronectin in Embryonic and Adult Human Tissues," Journal of Cell Science, 88:419-30, 1987.

A Tumor-associated Fibronectin Isoform Generated by Alternative Splicing of Messenger RNA Precursors

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Abstract. Fibronectin (FN) represents the mixture of a number of structurally different molecules (isoforms) whose make-up varies depending on the FN sources. FN from cultured transformed human cells has a very different isoform composition with respect to its normal counterpart. In fact, SV-40-transformed WI-38VAI3 human fibroblasts produce high levels of a FN isoform (B-FN) which is very poorly expressed in their normal, WI-38, counterpart. We have recently demonstrated that the B-FN isoform derives from a differential splicing pattern of the FN primary transcript which leads, in transformed cells, to a high level expression of the exon ED-B (Zardi, L., B. Carnemolla, A. Siri, T. E. Petersen, G. Paolella, G. Sebastio, and F. E. Baralle. 1987. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2337–2342). Here we report on the production and characterization of a monoclonal antibody (BC-1) which recognizes an epitope within the protein sequence coded for by the ED-B exon. This monoclonal antibody makes it possible to carry out immunohistochemical analysis of the distribution of the ED-B-containing FN isoform (B-FN) in human tissues. The results show that while in normal, adult, human tissues total FN has a widespread distribution, the B-FN isoform is restricted only to synovial cells, to some vessels and areas of the interstitium of the ovary, and to the myometrium. On the contrary, the B-FN isoform has a much greater expression in fetal and tumor tissues. These results demonstrate that, in vivo, different FN isoforms have a differential distribution and indicate that the B-FN isoform may play a role in ontogenesis and oncogenetic processes.

FIBRONECTIN (FN)[1] is a polymorphic high molecular mass adhesive glycoprotein present in soluble form in plasma and other body fluids and in insoluble form in the extracellular matrixes. Both these forms are dimers composed of subunits with a molecular mass of 250–280 kD made up of a series of repeating units of three types and joined by two disulfide bonds at the carboxyl terminus of the molecule. FN molecules are involved in various biological phenomena including the establishment and maintenance of normal cell morphology, differentiation, wound healing, cell migration, and adhesion (1, 2, 15, 41).

It has been previously demonstrated that FN polymorphism is at least partially caused by alternative splicing schemes in three regions of the primary transcript of a single gene which may generate 20 different FN subunit isoforms (18, 19, 29). One of these regions (IIICS, see Fig. 1 *A*) is between the last two type III homology repeats; a single exon is subdivided to yield five alternative patterns of splicing. Hynes and co-workers (34) showed that inclusion of the IIICS sequence contributed to the differences in size between the larger and smaller subunit of plasma FN. Humphries et al. (16) suggested that for some cell types, regulation of the adhesion-promoting activity of FN may occur by alternative RNA splicing in the IIICS area.

At the second region of variation (ED-A), a single exon encoding a complete type III repeat is either included or omitted from the mature mRNA. This variation is tissue specific and the ED-A sequence is absent in the mRNA of liver (10, 20, 21) which is the source of plasma FN (38). Using a rabbit antiserum to the rat ED-A segment, Paul et al. (31) demonstrated that this sequence is not present in plasma FN but is expressed in FN released by cultured fibroblasts and in FN from blood platelets. More recently, using mAbs Castellani et al. (8) and Borsi et al. (5) demonstrated that in FN from the tissue-culture medium of tumor-derived or SV-40-transformed human cells, the percentage of FN molecules containing the IIICS and ED-A sequences are higher than in FN from normal human fibroblasts.

At the third region of variation (ED-B), a single exon encoding a complete type III repeat is either included or omitted from the mature mRNA (14, 33, 44). The ED-B sequence presents two interesting peculiarities: (*a*) it is the more conserved FN region, 100 and 96% homology with rat and chicken FN, respectively (28, 44); and (*b*) this exon is highly expressed in transformed human cells, while it is barely de-

1. *Abbreviation used in this paper*: FN, fibronectin.

tectable in cultured normal human fibroblasts (44). These observations suggest that the ED-B sequence may introduce some specific biological function(s) into the FN molecule. A first step towards the understanding of the biological function(s) of the B-FN isoform is the study of its distribution in normal, pathological, and fetal tissues. In this paper we report the production and characterization of an mAb (BC-1) specific for the protein sequence coded for by the ED-B exon. Using this mAb we demonstrated that while this sequence is undetectable in plasma FN and in almost all normal human adult tissues, it is highly expressed in fetal and tumor tissues.

Materials and Methods

Cell Lines and Monoclonal Antibodies

All cell lines were grown in Eagle's minimum essential medium supplemented with 10% fetal calf serum (Flow Laboratories, Irvine, Scotland, UK). When FN had to be quantified in conditioned media, fetal calf serum was depleted of bovine FN by passage through a large capacity gelatin-Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden). mAbs specific for human FN were prepared as previously described (43) by fusion of P3U1 myeloma cells with splenocytes from mice immunized with FN from WI38VA13 or WI38 cells, or plasma. The characterization of mAbs IST-4 and IST-9 has been previously reported (5, 7, 36). The mAb 3E3 (32) specific to the cell-binding region of FN was a gift from M.D. Pierschbacher and E. Ruoslahti (La Jolla Cancer Research Foundation, La Jolla, CA). The radioimmunoassay was carried out as previously reported (45) and immunodot-blot analysis was performed using Bio-Rad Laboratories (Richmond, CA) equipment following the manufacturer's instructions.

Purification and Proteolytic Digestion of FN and Its Fragments

FN was purified from human plasma and from the conditioned media of cell lines as previously reported (46). Thermolysin (Protease type X; Sigma Chemical Co., St. Louis, MO) digestion of FN and cleavage of FN by *S*-cyanilation with 2-nitro-5-thiocyanobenzoic acid were carried out as described by Sekiguchi and Hakomori (35). The FN 120- and 110-kD fragments (domain 4, see Fig. 1 *A*) were purified from a FN–thermolysin digest (6 μg/mg of FN for 2 h at 22°C) by a hydroxyapatite (DNA grade; Bio-Rad Laboratories) chromatography column as previously described (6, 42). Complete separation of the 120-kD domain 4 from the 110-kD domain 4 was achieved using a DEAE–cellulose (Whatman Inc., Maidstone, England, UK) chromatography column. The two fragments, 35 and 85 kD, obtained by thermolysin digestion of the 120-kD domain 4 were separated by a hydroxyapatite chromatography column. SDS-PAGE and immunoblotting were carried out as described by Laemmli (22) and Towbin et al. (39).

Acrylamide, SDS, and other electrophoresis reagents were from Bio-Rad Laboratories. Nitrocellulose filters were from Schleicher and Schuell, Inc. (Dassel, West Germany). Rabbit antiserum to human FN was prepared as previously described (46).

Construction of cDNA Library in λgt 11 Phage

Total RNA was extracted from WI-38VA13 cells following the procedure of Chirgwin et al. (9) as modified by Freeman et al. (12). Poly(A) RNA was purified using an oligo(dT)–cellulose column according to the method described by Maniatis et al. (24). For cDNA preparation, a cDNA synthesis system purchased from Pharmacia Fine Chemicals was used following the manufacturer's instructions. cDNA, without further purification, was treated with Eco RI metilase (BioLabs, Beverly, MA), Eco RI linkers (BioLabs) were added, and the cDNA digested with Eco RI. cDNA was then ligated to λgt 11 arms using Stratagene (San Diego, CA) kits, for both ligation and packaging, following methods described by Huynh et al. (17). All enzymes used, unless otherwise specified, were from Boehringer Mannheim GmbH (Mannheim, West Germany).

Isolation and Analysis of FN cDNA Clones

A WI-38VA13 cDNA library, in the expression vector λgt 11, prepared as described above, was screened by an immunoenzymatic procedure (17) using the mAb BC-1 and an immunoenzymatic kit purchased from Promega Biotec (Madison, WI). Bacteriophage DNA was prepared from positive clones, and the Eco RI inserts were excised, subcloned into a pUC8 plasmid vector (11, 40), and sequenced by Maxam and Gilbert's method (27). All the positive clones obtained contain a FN insert with at least part of the ED-B sequence. One of these clones, λF2, contains the complete ED-B sequence and 315 bases upstream and 255 bases downstream. We constructed a clone λF6c by removing the region containing the ED-B sequence from the clone λF2 by Bsp MI and Ban II restriction enzymes (BioLabs) and substituting it with an identical fragment, except that it lacked the ED-B sequence. This fragment was obtained by the same restriction enzymes from the clone pFH154 (20) in which the ED-B sequence is not present. The clone pFH154 was a gift from Dr. F. E. Baralle (Istituto Sieroterapico Milanese, Milan, Italy). All the cloning and subcloning procedures were carried out according to Maniatis et al. (24).

For the immunoblotting analysis the β-galactosidase–FN fusion proteins were obtained from the clones using the following procedure: ~20,000 plaque-forming units were plated with *Escherichia coli* (Y 1090 strain) in 0.7% LB agar containing 0.5 mM isopropyl β-D-thiogalactopyranoside, 50 μg/ml ampicillin in 95-mm Petri dishes and incubated at 37°C overnight. Then 2 ml of Tris-HCl, pH 6.8, buffer, containing 2% SDS, 4% β-mercaptoethanol, 10% glycerol, was added to each dish and incubated at room temperature for 2 h with gentle agitation. The buffer was then removed, briefly centrifuged, and 20-μl samples of the supernatant were analyzed by SDS-PAGE.

Tissue Samples and Immunohistochemical Studies

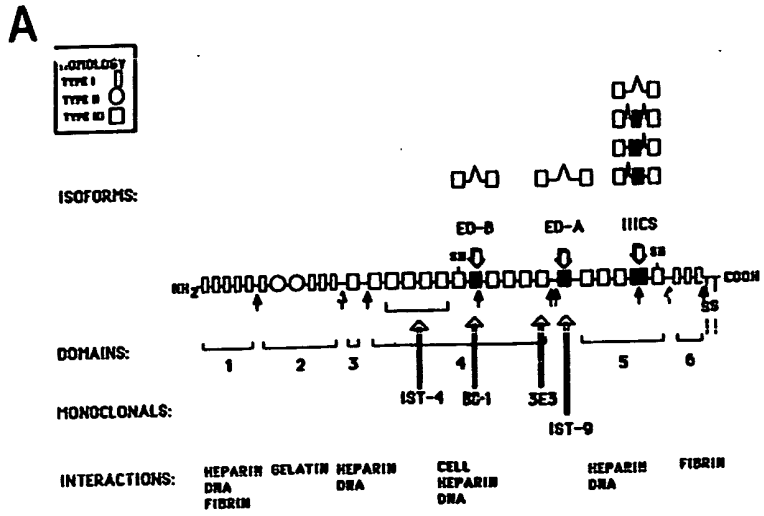
Normal and neoplastic tissues were obtained from surgical samples of patients free from chemo- and radiotherapy. Fetal tissues were taken from spontaneous abortions. Each sample was divided into two portions: one was processed for conventional histopathological diagnosis and the other was immediately snap-frozen in liquid nitrogen. 4-μm-thick cryostat sections were stained with 0.1% toluidine blue in PBS (20 mM Na-phosphate buffer, pH 7.3, 0.15 M NaCl) and additional frozen sections were used for indirect immunofluorescence and immunoperoxidase staining after fixation in cold absolute acetone for 10 min. To avoid the heterogeneous distribution within the tissues of FN isoforms being responsible for false negative findings, at least three nonconsecutive sections of the tissue were analyzed.

All indirect immunofluorescence and immunoperoxidase stains were made using mAbs as the hybridoma culture supernatant, rabbit anti–mouse Ig FITC-labeled antiserum (Cappel Laboratories, Cochranville, PA), and a commercially available avidine–biotine staining kit (Vector Laboratories, Inc., Burlingame, CA). The immunoenzymatic stain used 3-amino-9-ethylcarbazole as a chromogenic substrate and Mayer's hematoxylin as a counterstain followed by mounting in buffered glycerol.

Results

Generation of the mAb BC-1 Which Recognizes an Epitope Within the FN Sequence ED-B

mAbs obtained by fusion of splenocytes from mice immunized using FN from the culture medium of SV-40-transformed human fibroblasts WI-38VA13 were screened with a radioimmunoassay system using plasma FN, FN from WI-38VA13 cells, and FN from their normal counterpart WI-38 cells as antigens. We obtained a clone (BC-1) which released mAbs specific for FN from WI-38VA13 cells but which did not react with plasma FN and only reacted very weakly with FN from the normal human fibroblasts WI-38. We tested this mAb using the immunoblotting technique, with thermolysin digests of FNs from plasma, WI-38, and WI-38VA13 cells. In accordance with the data obtained using the radioimmunoassay system, the mAb BC-1 showed strong reaction with WI-38VA13 FN fragments, negative reaction with plasma FN fragments, and a barely detectable reaction with WI-38 FN fragments (Fig. 1 *B*). The immunoblot pattern obtained with the mAb BC-1 was compared with those of other mAbs specific to different human FN epitopes (Fig. 1 *B*). The staining pattern obtained with the mAb IST-9



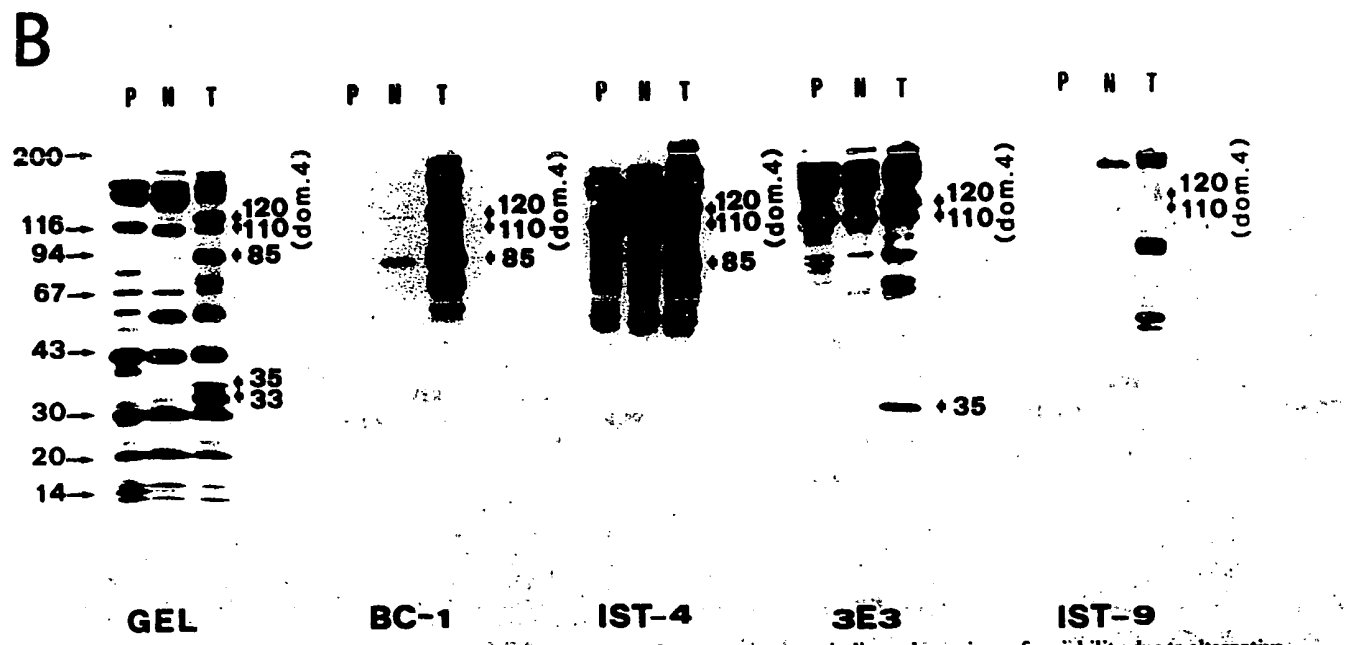
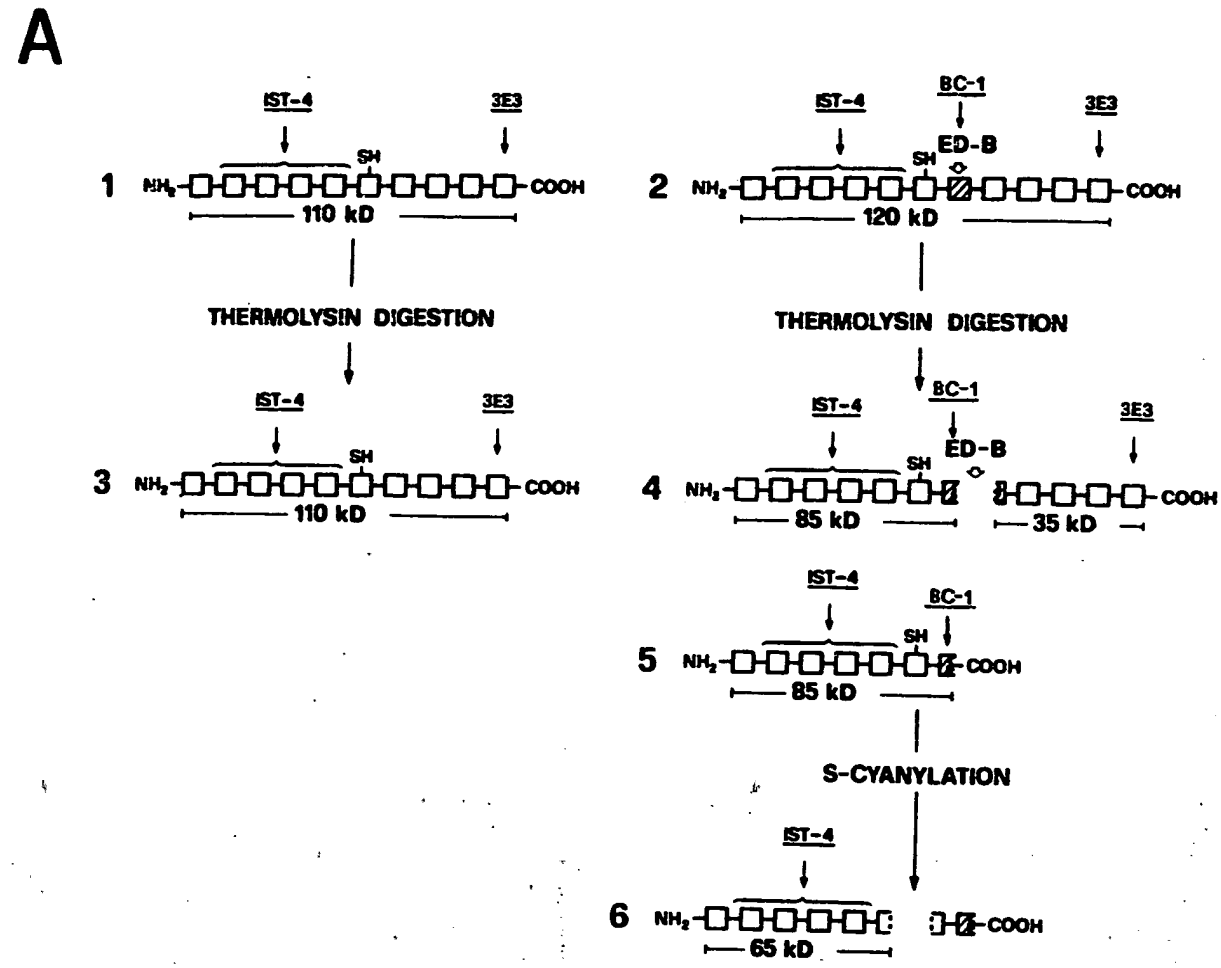


Figure 1. (A) Model of the domain structure of a subunit of human FN. Large open arrows indicate the regions of variability due to alternative splicing of the FN mRNA precursors. Black arrows indicate the thermolysin cleavage sites. Long open arrows indicate the sites where the epitopes recognized by various mAbs are located. The figure also indicates the internal homologies; macromolecules interacting with the various FN domains and the possible isoforms generated by alternative splicing. (*B*) On the left a 4–18% SDS-PAGE gradient of 20 μg each of plasma FN (*P*), FN from WI-38 normal human cells (*N*), and from SV-40-transformed human fibroblasts WI-38VA13 (*T*) digested by thermolysin (5 μg/mg of FN) for 2 h at 22°C is shown. The values on the left are the molecular masses (in kD) of the standards. The values on the right are the molecular masses (in kD) of the 110-kD domain 4 and of the four major fragments only present in thermolysin digests of FN from transformed cells. Immunoblots of a similar gel using the mAbs BC-1, IST-4, 3E3, and IST-9 are also shown. The two forms of the domain 4 120-kD fragment (containing the ED-B sequence) and 110-kD fragment (without the ED-B sequence) are indicated.

(which recognizes the ED-A sequence) was completely different from that obtained with the mAb BC-1 (Fig. 1 *B*). This rules out the hypothesis that the mAb BC-1 recognizes the ED-A sequence which is preferentially expressed in FN from transformed cells and absent in plasma FN (5). The mAb BC-1 reacts strongly with a 120-kD fragment, which is the cell-binding domain 4 containing the ED-B sequence (see Fig. 1 *B*) that is produced almost exclusively by transformed cells (44), but it does not react with the 110-kD fragment, which is the cell-binding domain 4 without the ED-B sequence (see Fig. 1 *B*). These data strongly suggested that the epitope recognized by the mAb BC-1 is within the ED-B sequence. The 110- and 120-kD cell-binding domain 4 were purified and digested with thermolysin (Fig. 2). While the 110-kD domain 4 is resistant to thermolysin, the digestion of the 120-kD domain 4 generates two fragments: a 85-kD fragment which contains almost the complete ED-B sequence in its carboxyl-terminal part and reacts with the mAb



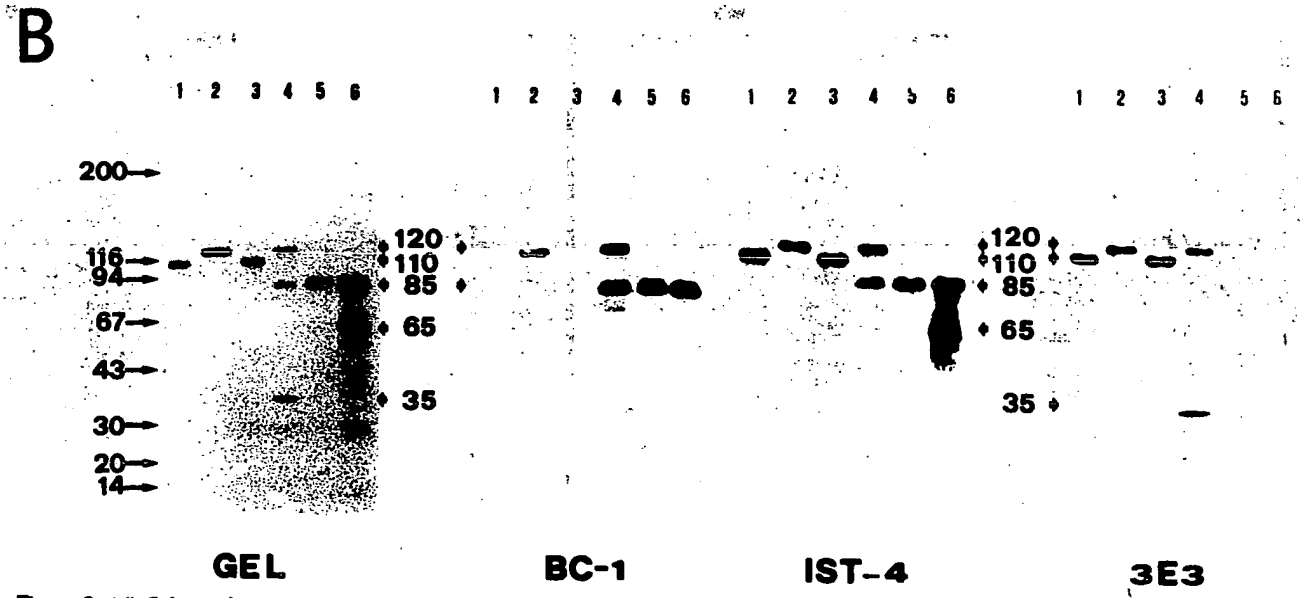


Figure 2. (*A*) Schematic representation of the thermolysin digestion of the 110-kD (domain 4 without the ED-B sequence) and 120-kD (domain 4 containing the ED-B sequence) fragments. The *S*-cyanilation cleavage of the 85-kD fragment obtained from the 120-kD d main 4 is also schematically represented. The epitopes reacting with the mAbs 3E3, IST-4, and BC-1 are indicated. The numbers on the left (*1–6*) indicate the corresponding lanes of gels and immunoblots shown in Fig. 2 *B*. (*B*) On the left, a 4–18% SDS-PAGE gradient of the purified 110-kD domain 4 (lane *1*) and 120-kD domain 4 (lane *2*); thermolysin digest of the 110-kD fragment (lane *3*); therm lysin digest of the 120-kD fragment (lane *4*); purified 85-kD fragment from the therm lysin digesti n of the 120-kD fragment (lane *5*) and the 85-kD fragment cleaved by *S*-cyanilation. The values on the left indicate the molecular masses, in kD, f the standards. The values on the right indicate the molecular masses, in kD, of the various FN fragments. On the right immun blots of similar gels using the mAbs BC-1, IST-4, and 3E3, respectively, are shown.

BC-1, and a 35-kD fragment (Fig. 2) (44). The 85-kD fragment was purified and its carboxyl-terminal part containing the ED-B sequence was cleaved out by *S*-cyanilation. This generated a 65-kD fragment which no longer reacted with the mAb BC-1 (Fig. 2), demonstrating that the epitope recognized by this mAb is within the FN fragment containing the ED-B sequence which was removed.

To confirm the hypothesis that the mAb BC-1 recognizes an epitope within the ED-B sequence we used β-galactosidase–FN fusion proteins containing or not containing the ED-B sequence. A WI-38VA13 library in the expression vector λgt 11 was screened by an immunoenzymatic procedure using the mAb BC-1. All the positive clones obtained contained a FN DNA insert with at least part of the ED-B sequence as demonstrated by DNA sequencing. In particular, the clone λF2 produces a β-galactosidase–FN fusion protein in which the FN part is composed of the complete ED-B sequence plus 105 amino acids at its amino-terminal and 85 amino acids at its carboxyl-terminal ends (see Fig. 5). As a negative control we constructed a clone, λF6c, (see Materials and Methods) expressing a β-galactosidase–FN fusion protein identical to that produced by the cloned λF2 except that it lacked the entire ED-B sequence (Fig. 3). As shown in Fig. 3, the mAb BC-1 reacts only with the fusion protein containing the ED-B sequence from the clone λF2, but does not react with the fusion protein without the ED-B sequence produced by the clone λF6c. This demonstrates that the epitope recognized by the mAb BC-1 is localized within the ED-B sequence.

Fig. 4 shows a dot-blot analysis of plasma FN, FNs from three different normal human fibroblast cell lines and four transformed or tumor-derived cell lines using the mAbs IST-4 (which recognizes all different FN isoforms) and BC-1 (which recognizes only the B-FN isoform). While the mAb IST-4 reacts in the same way with all different FNs, the mAb BC-1 shows a reaction only with FNs from the SV-40-transformed human fibroblasts WI-38VA13 and with the rhabdomyosarcoma cell line RD.

Distribution of the B-FN Isoform in Human Fetal, Adult, and Tumor Tissues

Table I summarizes the results of the immunohistochemical analysis of a variety of fetal and normal adult tissues using the mAbs BC-1, which recognizes only the ED-B-containing FN molecules, B-FN, and IST-4, which recognizes all different FN isoforms. In adult tissues, while total FN has a widespread distribution (See Table I and reference 37), the presence of the B-FN isoform is limited to the superficial synovial cells, the intima of some ovarian vessels, scattered areas of the ovarian interstitium, isolated areas of the basement membranes of the celomic epithelium, and to areas of the myometrium (Table I). On the contrary, in tissues from 8–12-wk old fetuses the B-FN isoform is much more represented. In fact, among the tissues tested, the only negative ones were kidney, liver, colon, and skin tissues (see Table II). In particular the B-FN isoform is present in the intima of the vessels of fetal brain cortex, stomach, jejunum, thymus, and lung. The mAb BC-1 also stains some areas of the submucosa of the stomach and jejunum, and the basement membranes of the stomach and of developing bronchia (Fig. 5). However, in tissues from older fetuses (22–26 wk old) only the basal portion of the gastric and duodenal glands were found positive. This suggests that the B-FN isoform undergoes a programmed expression during ontogenesis.

Table III summarizes the results of the immunohistochemical analysis of 165 different primary human tumors using the mAbs IST-4 and BC-1. About 38% of the tumors tested showed the presence of the B-FN isoform (Table III and Fig. 5). Within a given tumor type, the incidence of tumors showing the presence of the B-FN isoform was variable and no correlation was found with the degree of differentiation (colon adenocarcinomas) or the tumor histotype (breast tumors).

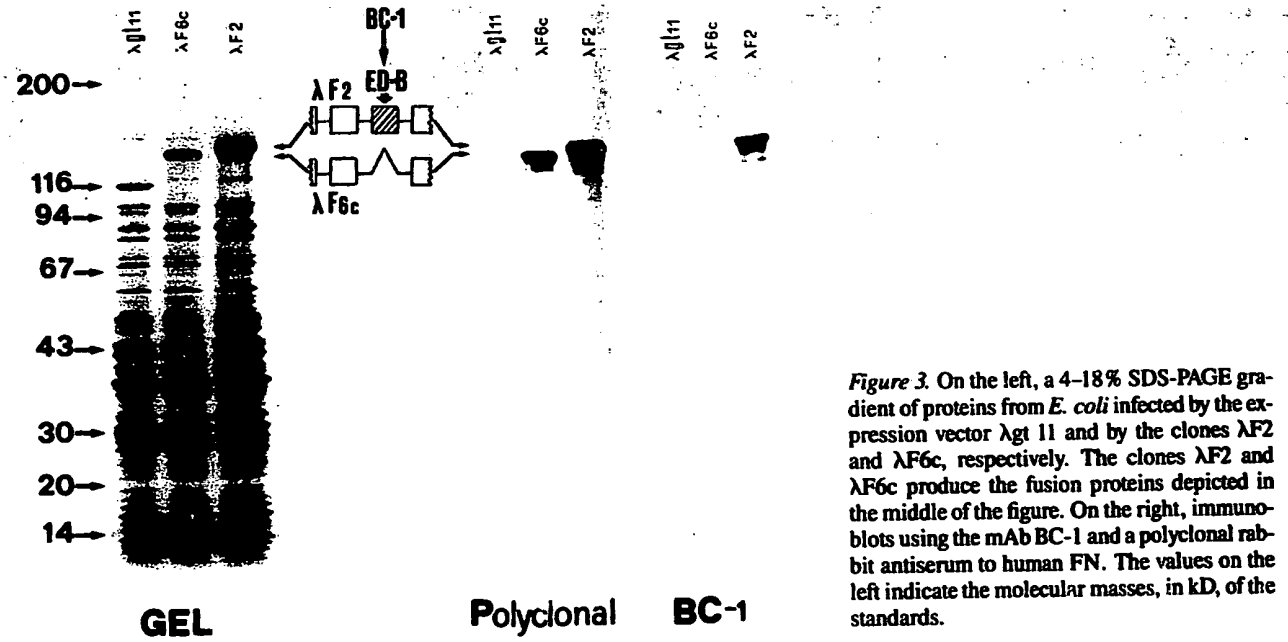


Figure 3. On the left, a 4–18% SDS-PAGE gradient of proteins from *E. coli* infected by the expression vector λgt 11 and by the clones λF2 and λF6c, respectively. The clones λF2 and λF6c produce the fusion proteins depicted in the middle of the figure. On the right, immunoblots using the mAb BC-1 and a polyclonal rabbit antiserum to human FN. The values on the left indicate the molecular masses, in kD, of the standards.

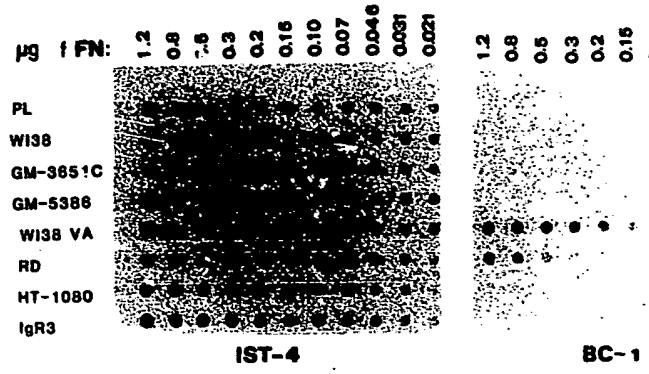


Figure 4. Dot-blot analysis of plasma FN (*PL*), and FNs from the following human cell lines: WI-38, embryonic lung fibroblasts; GM-3651-G, adult skin fibroblast; GM-5386, fetal skin fibroblasts; WI-38VAi3, SV-40-transformed embryonic lung fibroblasts; *RD*, from a rhabdomyosarcoma; HT-1080, from a fibrosarcoma; and IgR3, from a melanoma. The mAb IST-4 (*left*) recognizes all different FN isoforms while the mAb BC-1 (*right*) recognizes only the B-FN isoform.

The only exceptions were meningioma of psammomatous and meningotheliomatous type which were found to be consistently stained by the mAb BC-1. In some cases of renal, pulmonary, and colonic carcinomas, in which both normal and tumor tissues of the same patients could be studied, the B-FN isoform was found only in the transformed tissue. In almost all the specimens the B-FN isoform was confined to areas of variable extension of the tumor interstitium which surrounded or divided tumor cell nests of variable size. Staining was seldom found to outline scattered individual cells. A frequent feature was the presence of the B-FN isoform in the vascular intima which was never present in normal adult tissues with the exception of some ovarian vessels.

We also tested, with the mAb BC-1, three cutaneous scars (10–30 d old), and different benign and chronic inflammatory lesions: four breast fibroadenoma and one adenofibroma, five prostate hyperplasia, two fibrocystic disease samples, four gynecomastia, four urinary bladder and colorectal polyps, 14 intradermal nevus, two angioma, two ovarian cystoadenoma, two thyroid adenoma, one neurofibroma, two liver cirrhosis samples, and two hydatidosis cysts. All of them were found negative with the exception of the breast adenofibroma, which was variably positive in some interstitial areas.

Table I. Reactivity of Normal Adult Tissues with mAbs BC-1 and IST-4

	BC-1	IST-4
Brain (2)	−	+
Lung (3)	−	+
Breast (3)	−	+
Stomach (4)	−	+
Duodenum (3)	−	+
Colon (3)	−	+
Liver (2)	−	+
Pancreas (1)	−	+
Kidney (4)	−	+
Urinary bladder (2)	−	+
Prostate (2)	−	+
Testis (2)	−	+
Endometrium (2)	−	+
Spleen (2)	−	+
Lymphnode (2)	−	+
Skin (6)	−	+
Thymus (2)	−	+
Thyroid (2)	−	+
Meninges (2)	−	+
Skeletal muscle (1)	−	+
Choroid (2)	−	+
Retina (2)	−	+
Synovia (2)	+	+
Ovary (2)	+	+
Fallopian tubes (4)	−	+
Myocardium (1)	−	+
Choroid plexus (1)	−	+
Placenta (2)	−	+
Myometrium (4)	+	+
Celomic epithelium (2)	+	+

The number of individuals from which the tissues were obtained is indicated in parenthesis. The mAb BC-1 is specific only for the B-FN isoform, while the mAb IST-4 recognizes all different FN isoforms.

Discussion

In contrast to FN from normal human fibroblasts, in which the ED-B-containing molecules (B-FN) are barely detectable, in FN from SV-40-transformed cells the B-FN isoform represents ~30–40% of the molecules. This is due to a different splicing pattern of FN pre-mRNA which leads, in transformed cells, to high level expression of the exon ED-B (44).

In this paper we report on the production and characterization of an mAb (BC-1) which recognizes an epitope within the ED-B sequence. This localization has been established on the basis of the following results. (*a*) In immunoblotting experiments, the mAb BC-1 does not react with plasma FN; it shows a barely detectable reaction with FN from normal

Table II. Reactivity of Fetal (8–10-wk) Tissues with mAbs BC-1 and IST-4

	BC-1	IST-4
Brain cortex	+	+
Liver	−	+
Colon	−	+
Skin	−	+
Kidney	−	+
Stomach	+	+
Thymus	+	+
Lung	+	+
Jejunum	+	+

The mAb BC-1 is specific only for the B-FN isoform while the mAb IST-4 recognizes all different FN isoforms. In the same tissues and in those of the duodenum, spleen, pancreas, and urinary bladder of a 26-wk-old fetus, only the stomach and duodenum were found positive with the mAb BC-1.

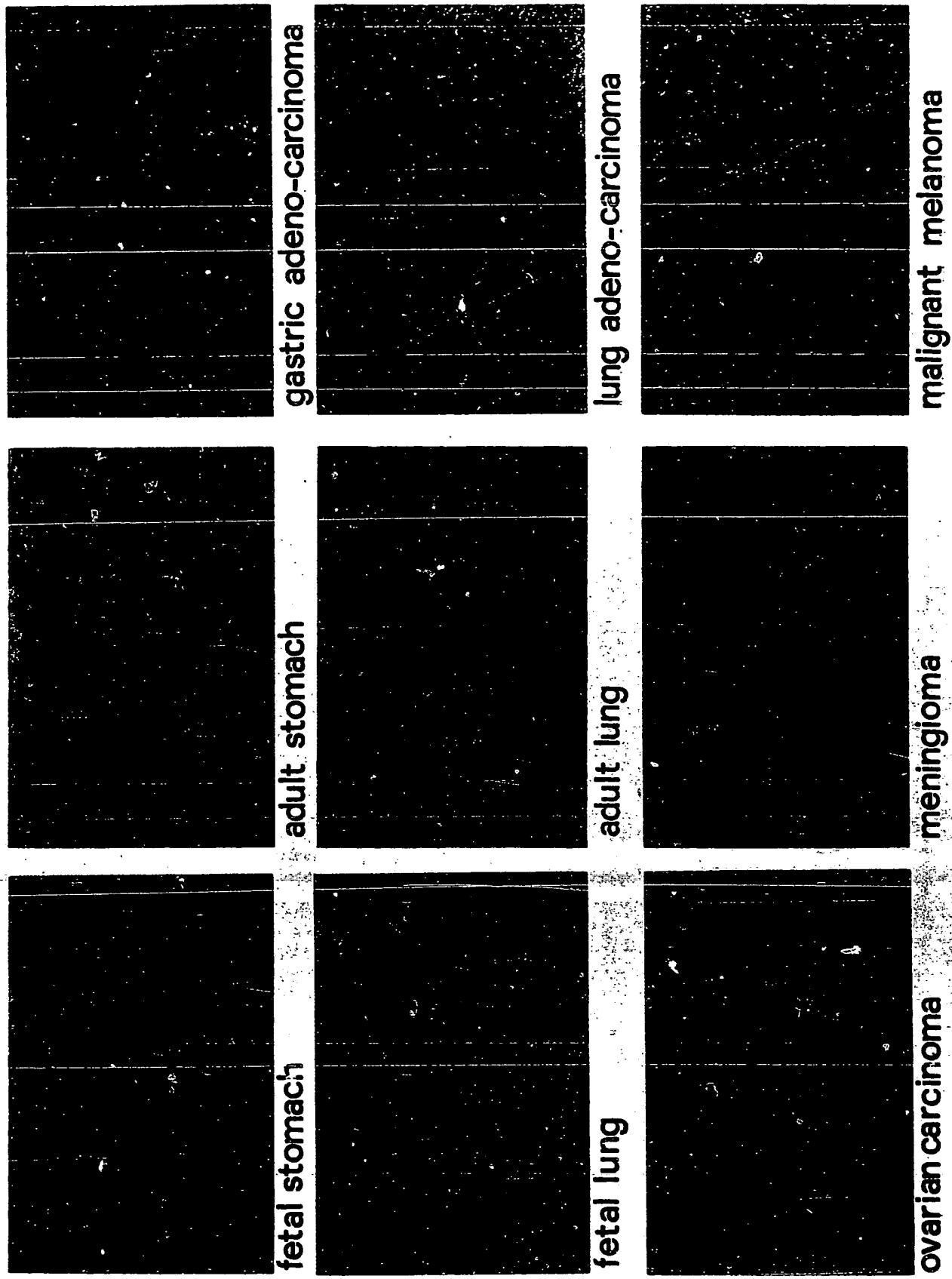


Figure 5. Study of the distributi n of the B-FN isoform in human fetal, adult, and tumor tissues by indirect immunoperoxidase staining using the mAb BC-1. The mAb BC-1 does not reac: with adult stomach but shows a positive reacti n along the basement membranes, (*arrowheads*) and in the wall of the submucosal vessels of the fetal stomach and in the septa of the gastric carcinoma. The mAb BC-1 does not react with the adult lung parenchyma but reacts with the basement membrane of the developing bronchia (*arrowheads*) and the wall of the submucosal capillaries of the fetal lung and the septa of the lung carcinoma. The mAb BC-1 shows positive reaction in the interstitium of the ovarian carcinoma, a punctuated pattern of staining with meningioma and outlines the boundaries of the cells in malignant melanoma. All these tissues showed a strong positive reaction when tested using the mAb IST-4 which recognizes all different FN isoforms. Bar, 10 μm.

Table III. Reactivity of the mAb BC-1 with Primary Tumors of Various Histotypes

Tumor type	Number positive/ Number tested
Stomach adenocarcinoma	3/7
Pancreas adenocarcinoma	0/3
Liver adenocarcinoma	2/7
Colon adenocarcinoma	2/10
Kidney clear cell carcinoma	4/8
Urinary bladder carcinoma	4/8
Prostate adenocarcinoma	1/5
Ovary adenocarcinoma	2/8
Endometrium adenocarcinoma	1/6
Skin carcinoma (mixed histotypes)	3/8
Thyroid adenocarcinoma	1/8
Lung carcinoma (mixed histotypes)	7/13
Breast adenocarcinoma	3/13
Skin and ocular melanoma	9/20
Brain tumor (mixed histotype)	4/13
Brain meningiomas	13/13
Sarcomas (mixed histotypes)	3/15
Total	62/165

All tumors tested were strongly positive with mAb IST-4 which recognizes all different FN isoforms. The mAb BC-1 recognizes only the B-FN isoform.

human fibroblasts but shows a very strong reaction with FN from SV-40-transformed fibroblasts (Fig. 1). These results are in agreement with those previously reported showing the absence of B-FN isoform in plasma, a very small amount in FN from normal fibroblasts, and a large amount in FN from SV-40-transformed fibroblasts (44). (*b*) Immunoblotting experiments, using different FN fragments, demonstrated that the mAb BC-1 reacts with an epitope localized in the central part of the 120-kD domain 4 (which contains the ED-B sequence) but does not react with the same domain lacking the ED-B sequence (110 kD) (Fig. 2). (*c*) The mAb BC-1 reacts with the fusion protein λF2 which contains the ED-B sequence, but does not react with the fusion protein λF6c which is identical to λF2 except that it lacks the ED-B sequence (Fig. 3); this also demonstrates that the mAb BC-1 recognizes a primary sequence epitope.

Previously, Matsuura and Hakomori (25), using the mAb FDC-6, suggested the existence of a domain present in FN from tumor and fetal tissues, but absent in FN from normal adult tissues. We exclude the possibility that the mAb FDC-6 recognizes an antigenic determinant within the ED-B sequence since the authors have localized this epitope within the IIICS sequence (see Fig. 1). Furthermore, the mAb FDC-6 does not recognize a primary sequence epitope (26).

Using the mAb BC-1 we compared the amount of the B-FN isoform in a panel of FNs from normal, tumor-derived, and transformed human cell lines using the dot-blot technique. The results demonstrate that the B-FN molecules are clearly detectable only in FNs from the SV-40-transformed, WI-38VA13 cell line and from the rhabdomyosarcoma cell line RD. Furthermore, the use of a more sensitive technique demonstrated that all the transformed cell lines tested produce FN in which the percentage of molecules containing the ED-B sequence is higher than in FN from normal human fibroblasts (Zardi, L., L. Bossi, and B. Carnemolla, manuscript in preparation). This increase of B-FN in transformed cells, with respect to normal cells. observed using the mAb BC-1 is in agreement with the data previously reported both at the mRNA and at the protein level (44). However, Schwarzbauer et al. (33) and Norton and Hynes (28) did not observe any significant increase in the relative amount of ED-B containing FN mRNA in transformed embryonic rat and chicken cells compared to the normal counterpart. At this moment, we cannot explain the differences between the above mentioned data and ours. Analysis, both at the protein and mRNA level, of larger panels of cultured cells from different species may be useful in clarifying these discrepancies. Furthermore, Castellani et al. (8) and Borsi et al. (5) have demonstrated that FNs from transformed or tumor-derived cells are composed of a population of molecules in which both the IIICS and ED-A sequences are expressed more than in FN from normal cells. The reason why tumor cells tend to express more of the FN sequences regulated by alternative splicing mechanisms has yet to be established.

The observed increased expression of the B-FN isoform in transformed, compared to normal, cell lines prompted us to study the distribution of the B-FN isoform in primary tumors of various histotypes and in normal adult and fetal tissues. In contrast to the widespread distribution of other FN isoforms, only a few normal adult tissues showed the presence of the B-FN isoform (see Table I). Unlike normal adult tissues, 38% of the 165 tumors tested displayed considerable levels of the B-FN isoform. It was distributed in the interstitium which surrounded the tumor cells, and in the vascular intima which in normal tissues was only detected in some ovarian vessels. The incidence of positive tumors was variable within type, with the exception of meningioma in which 13 cases out of 13 showed a positive reaction with the mAb BC-1. We have been unable to find any correlation between the presence of B-FN and the degree of tumor differentiation or histotype. This leaves the question open as to why some tumors show the presence of B-FN and others do not. Another unanswered question is whether the B-FN isoform is produced by the tumor cells or by the mesenchymal cells which surround the tumors. One possibility is that the tumor cells release factor(s) which stimulate the mesenchymal cells to synthesize the B-FN isoform. On the other hand, B-FN was absent from all the benign lesions, chronic inflammatory lesions, and cutaneous scars tested, with the single exception of a breast adenofibroma which displayed staining in only a few areas of the interstitium. In tissues from 8–10-wk-old fetuses, the B-FN isoform is more highly represented compared to adult tissues, but in those from 26-wk-old fetuses only the stomach and duodenum were found positive. These data are in agreement with those of Norton and Hynes (28) showing that B-FN mRNA was the predominant form in total embryo RNA from 2.5 to 11 d after fertilization.

These results clearly show a switching in the accumulation of the different FN isoforms associated with human ontogenesis and the reverse process associated with oncogenesis. The presence of the B-FN isoform in fetal tissues and its reappearance in tumors suggests that B-FN may play a role common to both situations and strongly indicates that transformation alters the splicing pattern of FN RNA towards that characteristic of fetuses.

Alternative RNA splicing is an important and widespread mechanism of gene regulation. The differential expression of exons into mature mRNAs is often under developmental

and/or tissue-specific regulation. It has been suggested that alternative splicing may be regulated by information encoded in the gene transcript (*cis*) but may require diffusible factor(s) (*trans*) that may be responsible for the developmental and/or tissue-specific regulation of splicing (3, 4, 13, 23, 30). Since transformation is associated with a deep modification of gene expression, it is not surprising that such a fine mechanism of gene regulation, as RNA splicing, may be altered in transformation. The alternative splicing of messenger RNA precursors in tumor cells and the biological function(s) f the sequences which are variably expressed may be fertile future research areas.

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References

1. Alitalo, K., and A. Vaheri. 1982. Pericellular matrix in malignant transformation. *Adv. Cancer Res.* 37:111–158.
2. Akiyama, S. K., and K. M. Yamada. 1982. Fibronectin in disease. *In* Connective Tissue Diseases. B. Wagner, R. Fleishmajer, and N. Kaufman, editors. Williams & Wilkins Co., Baltimore, MD. 55–96.
3. Breitbart, R. E., and B. Nadal-Ginard. 1987. Developmentally induced, muscle-specific trans-factors control the differential splicing of alternative and constitutive troponin T exons. *Cell.* 49:793–803.
4. Breitbart, R. E., A. Andreadis, and B. Nadal-Ginard. 1987. Alternative splicing: an ubiquitous mechanism for the generation of multiple protein isoforms from single genes. *Annu. Rev. Biochem.* 56:467–495.
5. Borsi, L., B. Carnemolla, P. Castellani, C. Rosellini, D. Vecchio, G. Allemanni, S. E. Chang, J. Taylor-Papadimitriou, H. Pande, and L. Zardi. 1987. Monoclonal antibodies in the analysis of fibronectin isoforms generated by alternative splicing of mRNA precursors in normal and transformed human cells. *J. Cell Biol.* 104:595–600.
6. Borsi, L., P. Castellani, E. Balza, A. Siri, C. Pellecchia, F. De Scalzi, and L. Zardi. 1986. Large-scale procedure for the purification of fibronectin domains. *Anal. Biochem.* 155:335–345.
7. Carnemolla, B., L. Borsi, L. Zardi, R. J. Owens, and F. E. Baralle. 1987. Localization of the cellular-fibronectin-specific epitope recognized by the monoclonal antibody IST-9 using fusion proteins expressed in E. Coli. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 215:269–273.
8. Castellani, P., A. Siri, C. Rosellini, E. Infusini, L. Borsi, and L. Zardi. 1986. Transformed human cells release different fibronectin variants than do normal cells. *J. Cell Biol.* 103:1671–1677.
9. Chirgwin, J. M., A. E. Przybla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.
10. Colombi, M., S. Barlati, A. Kornblihtt, F. E. Baralle, and A. Vaheri. 1986. A family of fibronectin mRNAs in human normal and transformed cells. *Biochim. Biophys. Acta.* 868:207–214.
11. Davis, L. G., M. D. Dibner, and J. F. Battey. 1986. Basic Methods in Molecular Biology. Elsevier Science Publishing, Co., Inc., New York. 222 pp.
12. Freeman, G. J., C. Clayberger, R. DeKruyff, D. S. Rosenblum, and H. Cantor. 1983. Sequential expression of new gene programs in inducer T-cell clones. *Proc. Natl. Acad. Sci. USA.* 80:4094–4098.
13. Green, M. R. 1986. Pre-mRNA splicing. *Annu. Rev. Genet.* 20:671–708.
14. Gutman, A., and A. R. Kornblihtt. 1987. Identification of a third region of cell-specific alternative splicing in human fibronectin mRNA. *Proc. Natl. Acad. Sci. USA.* 84:7179–7182.
15. Hormann, H. 1982. Fibronectin: mediator between cells and connective tissue. *Klin. Wochenschr.* 60:1265–1277.
16. Humphries, M. J., S. K. Akiyama, A. Komoriya, K. Olden, and K. M. Yamada. 1986. Identification of an alternatively spliced site in human plasma fibronectin that mediates cell type–specific adhesion. *J. Cell Biol.* 103:2637–2647.
17. Huynh, T. V., R. A. Young, and R. W. Davis. 1985. Constructing and screening cDNA libraries in λgt 10 and λgt 11. *In* DNA Cloning, a Practical Approach. Vol. I. D. M. Glover, editor. IRL Press, Ltd., Oxford, England. pp. 49–78.
18. Hynes, R. 1985. Molecular biology of fibronectin. *Annu. Rev. Cell Biol.* 1:67–90.
19. Kornblihtt, A. R., K. Umezawa, K. Vibe-Pedersen, and F. E. Baralle. 1985. Primary structure of human fibronectin: differential splicing may generate at least 10 polypeptides from a single gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 4:1755–1759.
20. Kornblihtt, A. R., K. Vibe-Pedersen, and F. E. Baralle. 1984. Human fibronectin: cell specific alternative mRNA splicing generates polypeptide chains differing in the number of internal repeats. *Nucleic Acid Res.* 12:5853–5868.
21. Kornblihtt, A. R., K. Vibe-Pedersen, and F. E. Baralle. 1984. Human fibronectin: molecular cloning evidence for two mRNA species differing by an internal segment coding for a structural domain. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:221–226.
22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
23. Leff, S. E., M. G. Rosenfeld, and R. M. Evans. 1986. Complex transcriptional units: diversity in gene expression by alternative RNA processing. *Annu. Rev. Biochem.* 55:1091–1117.
24. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.
25. Matsuura, H., and S. Hakomori. 1985. The oncofetal domain of fibronectin defined by monoclonal antibody FDC-6: its presence in fibronectins from fetal and tumor tissues and its absence in those from normal adult tissues and plasma. *Proc. Natl. Acad. Sci. USA.* 82:6517–6521.
26. Matsuura, H., K. Takio, K. Titani, T. Greene, S. B. Levery, M. E. K. Salyan, and S. Hakomori. 1988. The oncofetal structure of human fibronectin defined by monoclonal antibody FDC-6. *J. Biol. Chem.* 263: 3314–3322.
27. Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* 65:499–560.
28. Norton, P. A., and R. O. Hynes. 1987. Alternative splicing of chicken fibronectin in embryos and in normal and transformed cells. *Mol. Cell. Biol.* 7:4297–4307.
29. Owens, R. J., A. R. Kornblihtt, and F. E. Baralle. 1986. Fibronectin, the generation of multiple polypeptides from a single gene. *Oxf. Surv. Eucaryot. Genes.* 3:141–160.
30. Padgett, R. A., P. J. Grabowski, M. M. Konarska, S. Seiler, and P. A. Sharp. 1986. Splicing of messenger RNA precursors. *Annu. Rev. Biochem.* 55:1119–1150.
31. Paul, J. I., J. E. Schwarzbauer, J. W. Tamkun, and R. O. Hynes. 1986. Cell-type-specific fibronectin subunits generated by alternative splicing. *J. Biol. Chem.* 261:12258–12265.
32. Pierschbacher, M. D., E. G. Hayman, and E. Ruoslahti. 1981. Location of the cell-attachment site in fibronectin with monoclonal antibodies and proteolytic fragments of the molecule. *Cell.* 26:259–267.
33. Schwarzbauer, J. E., R. S. Patel, D. Fonda, and R. O. Hynes. 1987. Multiple sites of alternative splicing of the rat fibronectin gene transcript. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2573–2580.
34. Schwarzbauer, J. E., J. I. Paul, and R. O. Hynes. 1985. On the origin of species of fibronectin. *Proc. Natl. Acad. Sci. USA.* 82:1424–1428.
35. Sekiguchi, K., and S. Hakomori. 1983. Topological arrangement of four functionally distinct domains in hamster plasma fibronectin: a study with combination of S-cyanylation and limited proteolysis. *Biochemistry.* 22:1415–1422.
36. Sekiguchi, K., A. Siri, L. Zardi, and S. Hakomori. 1985. Differences in domain structure between human fibronectins isolated from plasma and from culture supernatants of normal and transformed fibroblasts. *J. Biol. Chem.* 260:5105–5114.
37. Stenman, S., and A. Vaheri. 1978. Distribution of a major connective protein, fibronectin, in normal human tissues. *J. Exp. Med.* 147:1054–1064.
38. Tamkun, J. W., and R. O. Hynes. 1983. Plasma fibronectin is synthesized and secreted by hepatocytes. *J. Biol. Chem.* 258:4641–4647.
39. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.
40. Vieira, J., and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertion, mutagenesis and sequencing with synthetic universal primers. *Gene (Amst).* 19:259–268.
41. Yamada, K. M. 1983. Cell surface interactions with extracellular materials. *Annu. Rev. Biochem.* 52:761–799.
42. Zardi, L., B. Carnemolla, E. Balza, L. Borsi, P. Castellani, M. Rocco, and A. Siri. 1985. Elution of fibronectin proteolytic fragments from a hydroxyapatite chromatography column. *Eur. J. Biochem.* 146:571–579.
43. Zardi, L., B. Carnemolla, A. Siri, L. Santi, and R. S. Accolla. 1980. Somatic cell hybrids producing antibodies specific to human fibronectin. *Int.*

J. Cancer. 25:325–329.
44. Zardi, L., B. Carnemolla, A. Siri, T. E. Petersen, G. Paolella, G. Sebastio, and F. E. Baralle. 1987. Transformed human cells produce a new fibronectin isoform by preferential alternative splicing of a previously unobserved exon. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:2337–2342.
45. Zardi, L., A. Siri, B. Carnemolla, L. Santi, W. D. Gardner, and S. O. Hoch. 1979. Fibronectin: a chromatin-associated protein? *Cell.* 18: 649–657.
46. Zardi, L., A. Siri, B. Carnemolla, E. Cosulich, G. Viale, and L. Santi. 1980. A simplified procedure for the preparation of antibodies to serum fibronectin. *J. Immunol. Methods.* 34:155–165.

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Thorpe et al., "Selective Killing of Proliferating Vascular Endothelial Cells by an Anti-Fibronectin Receptor Immunotoxin," 16th LH Gray Conference, University of Manchester Institute of Science and Technology, Sep. 17-21, 1990.

Other Reference Publication (89):
Aoyagi, "Distribution of Plasma Fibronectin in the Metastatic Lesion of Cancer: Experimental Study by Autoradiography," Thrombosis Research, 49:265-75, 1988.

Other Reference Publication (96):
Burton-Wurster, et al., "Expression of the Ed B Fibronectin Isoform in Adult Human Articular Cartilage," Biochemical and Biophysical Research Communication 165(2): 782-87, 1989.

Other Reference Publication (98):
Carnemolla et al., "A Tumor-associated Fibronectin Isoform Generated by Alternative Splicing of Messenger RNA Precursors," The Journal of Cell Biology, 108:1139-48, 1989.

Other Reference Publication (99):
Carnemolla et al., "The Inclusion of the Type III Repeat ED-B in the Fibronectin Molecule Generates Conformational Modifications that Unmask a Cryptic Sequence," The Journal of Biological Chemistry, 267(34):245898-92, 1992.

Other Reference Publication (100):
Carnemolla, et al., "Phage Antibodies with Pan-Species Recognition of the Oncofoetal Angiogenesis Marker Fibronectin ED-B Domain," Int. J. Cancer 68:397-505, 1996.

Other Reference Publication (101):
Castellani, et al., "The Fibronectin Isoform Containing the ED-B Oncofoetal Domain: A Marker of Angiogenesis," Int. J. Cancer, 59:612-18, 1994.

Other Reference Publication (104):
Farnoud, et al., "Fibronectin Isoforms Are Differentially Expressed in Normal and Adenomatous Human Anterior Pituitaries," Int. J. Cancer, 61:27-34, 1995.

Other Reference Publication (108):
Kaczmarek, et al., "Distribution of Oncofetal Fibronectin Isoforms in Normal Hyperplastic and Neoplastic Human Breast Tissues," Int. J. Cancer, 58:11-16, 1994.

Other Reference Publication (112):
Linnala, et al., "Human Amnion Epithelial Cells Assemble Tenascins and Three Fibronectin Isoforms in the Extracellular Matrix," FEBS, 314(1,2):74-78, 1993.

Other Reference Publication (115):
Oyama, et al., "Coordinate Oncodevelopmental Modulation of Alternative Splicing of Fibronectin Pre-Messenger RNA at ED-A, ED-B and CS1 Regions in Human Liver Tumors," Cancer Research 53:2005-11, 1993.

Other Reference Publication (116):
Peters, et al., "Expression of the Alternatively Spliced EIIB Segment of Fibronectin," Cell Adhesion and Communication, 3:67-89, 1995.

Other Reference Publication (126):
Saiki et al., "Inhibition of Tumor Angiogenesis by a Synthetic Cell-Adhesive Polypeptide Containing the Arg-Gly-Asp (RGD) Sequence of Fibronectin, Poly(RGD)," Jpn. J. Cancer Res. 81:668-75, 1990.

Other Reference Publication (128):
Schwarzbauer, "Alternative Splicing of Fibronectin: Three Variants, Three Functions," BioEssays 13(10)527-33, 1991.

Other Reference Publication (133):
Ueda, et al., "Selective Distribution of Fibronectin to a Tumor-Cell Line," Cancer Letters 31:261-65, 1986.

Other Reference Publication (134):
Vartio, et al, "Differential Expression of the ED Sequence-Containing Form of Cellular Fibronectin in Embryonic and Adult Human Tissues," Journal of Cell Science, 88:419-30, 1987.

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Coordinate Oncodevelopmental Modulation of Alternative Splicing of Fibronectin Pre-Messenger RNA at ED-A, ED-B, and CS1 Regions in Human Liver Tumors[1]

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ABSTRACT

The molecular diversity of fibronectin arises from alternative RNA splicing at regions termed ED-A, ED-B, and IIICS. We investigated the splicing patterns of fibronectin pre-mRNA at both ED-B and IIICS regions in various human liver tissues with an emphasis on the expression of the alternative cell adhesive site CS1 within the IIICS region. The relative abundance of the fibronectin mRNA containing the CS1 sequence was significantly increased in both fetal and cancerous liver tissues, although it was not affected in nonmalignant tissues with chronic hepatitis and cirrhosis. Similarly, the relative abundance of the fibronectin mRNA containing the ED-B region was also increased in both fetal liver and liver tumors, showing a close parallelism with the splicing pattern at the ED-A region. Immunohistochemical examination of cancerous liver tissues with monoclonal antibodies directed to the ED-A and ED-B segments revealed that the fibronectin isoforms containing these extra peptide segments were specifically deposited in the tumor nodules. Other genes encoding kininogen, γ chain of fibrinogen, and β-amyloid protein precursor, all of which had been shown to be alternatively processed, did not show any significant alteration in the splicing pattern in cancerous liver tissues. These results indicate that the alternative splicing of fibronectin pre-mRNA at the ED-A, ED-B, and IIICS regions is coordinately modulated in both fetal and cancerous liver tissues toward inclusion of the extra peptide segments and that not all but only selected genes are susceptible for "fine tuning" of alternative RNA splicing in cancerous liver tissues.

INTRODUCTION

FNs[4] are adhesive glycoproteins (M_r 250,000–270,000) present in various body fluids and extracellular matrix. The monomer subunits are connected by disulfide bonds near the carboxyl terminus to yield dimeric and multimeric molecules (see Fig. 1). FN subunits are composed of three internally homologous repeats, termed types I, II, and III (1). These homologous repeats are assembled into a series of functional domains capable of binding to such ligands as fibrin, heparin, certain types of bacteria, and cell surface adhesion receptors termed integrins (see Refs. 2–4 for recent reviews). Through binding to these extracellular ligands, FNs play an important role in the attachment and spreading of cells and in three-dimensional organization of extracellular matrices (see the reviews above).

FNs prepared from various cells and tissues have similar properties but differ in their subunit size (5, 6). Recent studies have revealed that multiple FN isoforms are generated from a single pre-mRNA by alternative splicing at three distinct regions referred to as ED-A, ED-B, and IIICS (7–11). ED-A and ED-B regions, both representing extra type III repeats, are encoded by individual cassette exons which are alternatively excluded from the mature mRNA (10–13). Alternative RNA splicing at each of these regions gives rise to two isoforms with and without respective ED sequences. IIICS is encoded as a part of the exon encoding the 5′ half of the last type III repeat which contains internal donor and acceptor sites for optional splicing within the exon (14, 15). Five mRNA variants (Variants 1–5) arise from a single pre-mRNA by alternative splicing at this region. Up to 20 different FN isoforms could be generated, i. . . the combinations among alternatively spliced variants at the ED-A, ED-B, and IIICS regions are possible.

Although the physiological roles of the ED-A and ED-B segments have been only poorly understood, the amino-terminal 25 residues of the IIICS region, often referred to as CS1, were shown to have a selective adhesive affinity for lymphoid cells and some tumor cells (16, 17). Another cell adhesive site was also identified within the carboxyl-terminal 31 residues of the IIICS region and referred to as CS5, although the adhesive activity of the CS5 segment was much weaker than that of the CS1 segment (16). It has been shown that integrin $\alpha_4\beta_1$, a novel FN receptor distinct from the integrin $\alpha_5\beta_1$ recognizing the Arg-Gly-Asp sequence within FN, binds specifically to the CS1 segment (17, 18). The CS1 region functions as an alternative cell attachment signal for certain cell types and plays an important role in cell adhesion and migration during embryogenesis and tumor metastasis (19, 20).

Alternative splicing at ED-A, ED-B, and IIICS regions has been shown to be regulated in a tissue-specific and developmental stage-specific manner (8, 21–23). We have reported that the tissue-specific alternative RNA splicing at the ED-A region is deregulated in malignant liver tumors so as to increase ED-A-containing (ED-A$^+$) FN mRNA (24). The increased expression of the ED-A$^+$ mRNA closely correlated with the malignancy of the liver tumors. The deregulated alternative splicing at the ED-B region was also observed in lung cancer tissues (25).

Recently we found that alternative pre-mRNA splicing of tenascin, another major component of extracellular matrix, was also deregulated in lung cancer tissues (26). These observations raise the questions of whether the expression of the ED-B region and/or the CS1 region is also perturbed during carcinogenesis and whether the alternative splicing of other hepatic gene transcripts is also deregulated in the same manner. In the present investigation, we analyzed the patterns of alternative splicing at both the ED-B and IIICS regions in various human liver tissues in order to better understand the *in vivo* regulation of ED-B and CS1 expression. We present evidence that the expression of the three alternatively spliced regions is coordinately modulated in the liver during organogenesis and carcinogenesis.

MATERIALS AND METHODS

Nuclease S1 was purchased from Boehringer Mannheim Yamanouchi (Tokyo, Japan). All DNA-modifying enzymes and pUC119 were from Takara Shuzo (Kyoto, Japan). [α-^{32}P]dCTP was from Amersham. pGEM3 was obtained from Promega (Madison, WI). All oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer (Foster City, CA) using the phosphoramidite method. Human β-actin cDNA was purchased from Wako Pure Chemical Industries (Osaka, Japan).

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[4] The abbreviations used are: FN, fibronectin; IIICS, type III connecting segment; HCC, hepatocellular carcinoma; APP, β-amyloid protein precursor; cDNA, complementary DNA.

Tissues. HCC tissues as well as the surrounding nontumorous tissues were obtained from the surgically resected specimens for pathological examination. The tissue specimens were immediately frozen at −85°C after surgical resection. Tissue specimens with chronic hepatitis and cirrhosis were obtained from the nontumorous area distal from the tumor nodules. Fetal liver tissues were obtained from fetuses of 16–27-week gestations at autopsy after therapeutic abortions. Informed consent was obtained from parent(s) for examination of the fetal tissues according to the *Guidelines for Experiments Using Human Organs and Tissues* issued by the Japanese Association of Obstetrics and Gynecology. The numbers of each tissue specimens obtained from different individuals were as follows: normal adult liver, 5; fetal liver, 5; liver with chronic hepatitis, 6; liver cirrhosis, 10; hepatocellular adenoma, 1; Type 1 HCC, 7; Type 2 HCC, 10; HCC transplanted in nude mice, 2. All HCCs examined in this investigation were less than or equal to 3 cm in diameter and were classified into two groups on the basis of absence (Type 1) or presence (Type 2) of extranodular tumor growth (27).

Preparation of RNA. Total RNA was prepared from 0.5–2.0 g of frozen tissue as described previously (22, 28).

Probe DNA for Nuclease S1 Protection Analysis. The probe DNAs for nuclease S1 protection analysis of the ED-A and ED-B regions were prepared as described (25). The probe DNA for the IIICS region was prepared from FN cDNA by PCR amplification using 5′-ACCGTGTGGGTACAGGTG-3′ (corresponding to bases 852–870 of the FN cDNA sequence reported in Ref. 8) and 5′-GTCACAGAGGCTACTAT-3′ (corresponding to bases 1280–1296 in Ref. 8) as forward and reverse primers, respectively. cDNA synthesis and subsequent PCR reaction were performed as described previously (26). The amplified DNA was electrophoresed on 6% polyacrylamide gel, and the cDNA fragment with an expected size (*i.e.*, 444 base pairs) was recovered from the gel and phosphorylated at the 5′ end with T4 polynucleotide kinase. The IIICS cDNA fragment was subcloned to pUC119 at the *Sma*I sites. The insert was then excised by double digestion with *Hpa*II and *Hind*III to give rise to a 448 base fragment, of which the 3′ 35 bases were derived from the polylinker region of pUC119.

The probe DNA for human kininogen was prepared from the kininogen cDNA HTPI1529 (29), which was kindly provided by Dr. Iwao Ohkubo (Shiga Medical College). The plasmid was double digested with *Hind*III and *Hinf*I, and the resulting 433-base pair fragment was purified upon agarose gel electrophoresis. The 3′ recessed end was filled in with the Klenow fragment of DNA polymerase I. The DNA fragment was then digested with *Sau*3AI, and the resulting fragment (169 base pairs) was subcloned into the *Bam*HI/*Sma*I site of pGEM3. The resulting plasmid was digested with *Sau*3AI and *Pvu*II to yield the 229-base pair fragment, of which the 5′ 169 bases were derived from HTPI1529 and the 3′ 60 bases were from pGEM3.

The DNA probe for the γ chain of human fibrinogen (bases 1271–1392; Ref. 30) was prepared from the cDNA clone pHIγ2 (kindly provided by Dr. D. W. Chung, University of Washington) by PCR amplification using 5′-ACTTGGAAAACCCCGGTG-3′ and 5′-ACGTCTCCAGCCTGTTT-3′ as the forward and reverse primers. The amplified cDNA fragment was recovered from the gel and phosphorylated at the 5′ end with T4 polynucleotide kinase. The fibrinogen γ chain cDNA fragment was subcloned to pUC119 linearized by digestion with *Sma*I. The resulting plasmid was digested with *Hpa*II and *Hind*III to yield a 146-base pair fragment, of which the 3′ 35 bases were derived from the polylinker region of pUC119.

The DNA probe for human APP was prepared as described previously (31).

Probe DNAs for Quantitation of FN and β-Actin mRNAs. The DNA probes for the quantitation of FN and β-actin mRNAs were prepared from the 234-base pair *Bam*HI/*Pvu*II fragment of human FN cDNA clone pFH111 (8) and the 443-base pair *Hinf*I fragment of human β-actin cDNA, respectively, as described previously (26).

Preparation of 3′ End-labeled DNA Probe. The DNA probes were labeled at the 3′ end by the end-filling reaction with the Klenow fragment of DNA polymerase I using [α-^{32}P]dCTP and subjected to strand separation in order to recover the ^{32}P-labeled antisense strand DNA.

Nuclease S1 Protection Analysis. Nuclease S1 protection analysis was performed according to the method of Berk and Sharp (32). The 3′ end-labeled, single-stranded DNA probe was hybridized with 5–10 × 10^3 cpm of RNAs extracted from various liver tissues in 30 μl of 40 mM piperazine-*N,N*′-bis(2-ethanesulfonic acid) buffer (pH 6.4) containing 1 mM EDTA, 0.4 M NaCl, and 80% formamide at 43°C (probe for ED-B region), 49°C (probes for kininogen, γ chain of fibrinogen, and APP), or 53°C (probes for ED-A and IIICS region) for 18 h. The DNA/RNA hybrids were digested with 800 units of nuclease S1 at 37°C for 30 min as described (24, 25). The nuclease-resistant fragments were analyzed by electrophoresis on 6% polyacrylamide sequencing gels containing 7 M urea and subsequent autoradiography. Relative radioactivities of the nuclease-resistant fragments were determined by a Fujix Bioimage Analyzer BA100 using phosphor imaging plates.

For quantitation of the expression level of total FN mRNA, the 234-base pair *Bam*HI/*Pvu*II fragment of pFH111 was hybridized to RNA together with the β-actin DNA probe and subjected to digestion as described above. The level of FN mRNA was determined from radioactivities associated with the protected fragment after normalization against radioactivities associated with the protected β-actin DNA probe.

Immunohistochemistry. Fresh tissue specimens were fixed in 100% cold acetone at 4°C and embedded in paraffin. Thin sections were cut at a thickness of 3 μm and stained with monoclonal antibodies IST-9 and BC-1 (generous gifts from Dr. Luciano Zardi, Istituto Nazionale per la Ricerca sul Cancro, Genoa), specific to the ED-A and ED-B peptide segments, respectively (33, 34), by using the avidin-biotin complex method.

RESULTS

Oncodevelopmental Regulation of CS1 Expression in Liver Tissues. Alternative splicing at the IIICS region potentially generates five mRNA variants (see Fig. 1). Alternative splicing at this region was analyzed by nuclease S1 protection analysis using as a probe the antisense strand cDNA covering the 5′ 335 bases of the IIICS sequence. The probe DNA and its putative fragments protected by five mRNA variants are schematically illustrated in Fig. 2. The Variant 1 mRNA containing both CS1 and CS5 sequence should fully protect the probe DNA except for the 5′ 35 bases derived from the cloning vector. The Variant 2 mRNA containing the CS1 but lacking the CS5 sequence should partially protect the probe DNA due to mismatching after the central 192-base pair sequence. Other mRNA variants, Variants 3, 4, and 5, which are devoid of the CS1 sequence, should protect only the 5′ 78 bases common to all variant mRNAs. Although this probe cannot individually quantify the relative abundance of the Variant 3, 4, and 5 mRNAs, the overall expression of the FN mRNAs containing (*versus* lacking) the CS1 sequence can be estimated with this probe.

Nuclease S1 protection analysis of total RNA extracted from various human liver tissues with the CS1 probe DNA yielded all three of the expected fragments at different proportions (data not shown). The relative radioactivities of the three fragments thus generated are summarized in Fig. 3. The Variant 1 mRNA comprised no more than 10% of the total FN mRNA pool in normal adult liver, noncancerous liver with chronic hepatitis, cirrhosis, benign adenoma, and HCCs. There was no significant difference in the percentage of the Variant 1 mRNA between Type 1 and Type 2 HCCs, the latter of which was defined as HCC with extranodular tumor growth and considered to be more malignant than the former. The Variant 1 mRNA comprised slightly more than 20% of the total FN mRNA pool in fetal liver.

In contrast to the Variant 1 mRNA, the percentage of the Variant 2 mRNA was significantly higher in both fetal and malignant liver tissues than in nonmalignant adult tissues (Fig. 3*B*). The Variant 2 mRNA accounted for 40–50% of the total FN mRNA pool in normal and nonmalignant liver tissues, whereas the same mRNA species comprised 60%, 59%, and 63% in fetal liver, Type 1 HCC, and Type 2 HCC, respectively. No statistically significant difference was observed between Type 1 and 2 HCCs. Histological examination indicated that the increased expression of the Variant 2 mRNA in HCCs was not due to the outgrowth of fibroblasts or infiltration of macrophages, but rather to the deregulated alternative splicing of FN pre-mRNA. This was further supported by the observation that HCC tumors transplanted into nude mice, which were free from human

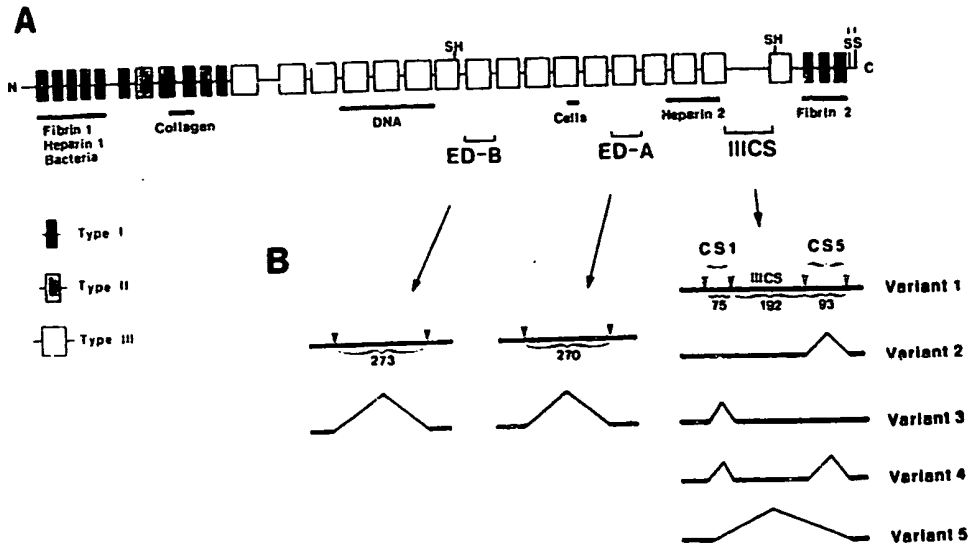

Fig. 1. Fibronectin structure and variants. *A*, molecular structure of FNs. FN polypeptides are composed of three types of internal homology repeats: type I (■), type II (▯), and type III (▯). Binding sites to various extracellular ligands are indicated by *bold underlines*. *B*, variations of FN mRNAs. Multiple FN isoforms differing in the structure at the ED-A, ED-B, and IIICS regions arise from alternative RNA splicing. Two mRNA variants are generated by either inclusion or exclusion of the ED-A and ED-B exons. Five variants are generated by exon subdivision at the IIICS region.

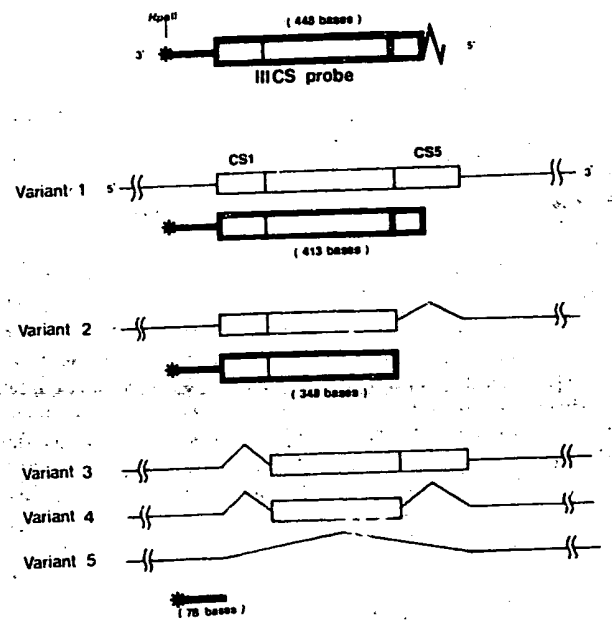

Fig. 2. Schematic representation of the probe DNA and the putative fragments protected by FN mRNA variants at the IIICS region. The probe DNA (*bold lines*) contains extra nucleotides derived from the cloning vector at the 5′ end (*wavy lines*).

fibroblasts or macrophages, also expressed an elevated level of the Variant 2 mRNA.

Expression of FN mRNAs lacking the CS1 sequence, *i.e.*, Variants 3, 4, and 5, was inversely correlated with that of Variant 2 mRNA, which contained the CS1 sequence (Fig. 3*C*). Variant 3, 4, and 5 mRNAs (collectively termed CS1$^-$ mRNAs) were abundant in normal and noncancerous tissues, whereas the percentage of these mRNA species within the total FN mRNA pool decreased in both fetal and malignant liver tissues. These results indicated that the expression of the cell adhesive CS1 segment in FN is oncodevelopmentally regulated in the liver.

Coordinate Deregulation of Alternative Splicing at ED-A, ED-B, and IIICS Regions in Malignant Liver Tumors. Alternative splicing at two ED exons, *i.e.*, ED-A and ED-B, generates two types of mRNA species, one with and the other without the ED exons (see Fig. 1). Previously we reported that alternative splicing at the ED-A region was deregulated in the malignant liver tumors (24). In order to extend the observation to the expression of the ED-B region, we compared the splicing patterns of FN pre-mRNA at both ED-A and ED-B regions in various liver tissues. Nuclease S1 protection analysis with specific DNA probes for ED-A and ED-B regions revealed a close parallelism in the alternative splicing patterns at these two ED regions (Fig. 4). Thus, the ED-B$^+$ mRNA was barely detectable in normal adult liver, while more than 20% of FN mRNA in the fetal liver contained the ED-B exon. In noncancerous liver tissues such as those with chronic hepatitis, cirrhosis, and benign adenoma, the percentage of ED-B$^+$ mRNA was less than 5%, indicating that the alternative splicing at the ED-B region was not affected in these nonmalignant diseases.

In contrast, malignant HCCs, both Types 1 and 2, expressed a significantly elevated percentage of the ED-B$^+$ mRNA, as was the case with the ED-A$^+$ mRNA. Further support for the elevated expression of the ED-B$^+$ mRNA in HCCs was obtained with HCC transplants in nude mice where nearly one-half of the total FN mRNA contained the ED-B exon. More pronounced expression of the ED-B$^+$ mRNA in HCC transplants than in primary tumors could be due to the phenotypic drift associated with successive passage of the tumors in nude mice. These results, taken together, clearly indicated that alternative splicing of FN pre-mRNA at the ED-A and ED-B regions was coordinately regulated in liver tissues in an oncodevelopmental manner. It should be also noted that there was a close parallelism among the splicing patterns at all three alternatively spliced regions, *i.e.*, ED-A, ED-B, and IIICS, in cancerous and noncancerous liver tissues, indicating that alternative splicing at these regions was simultaneously deregulated in liver cancer.

Despite the clear alterations in the alternative splicing patterns of FN pre-mRNA, the level of the total FN mRNA expressed was not significantly different between noncancerous and cancerous liver tissues, including HCC transplanted in nude mice (Fig. 5). These results

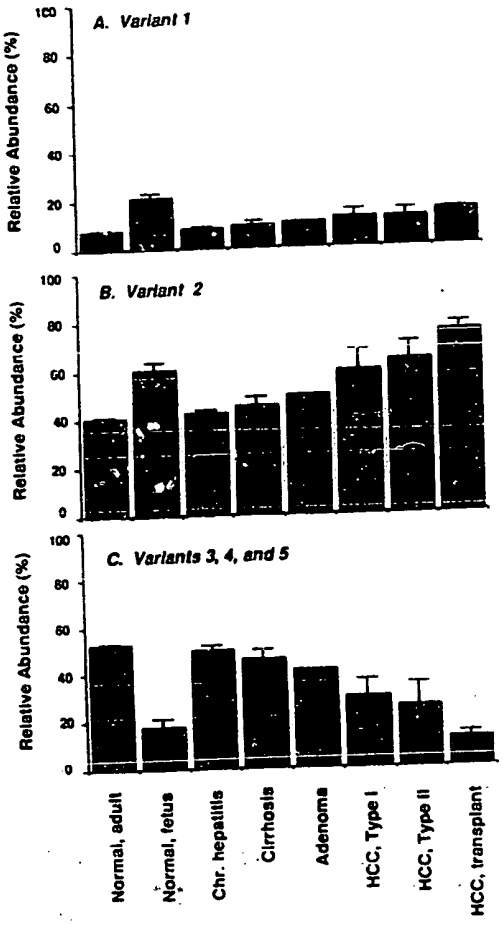

Fig. 3. Relative abundance of IIICS Variant mRNAs in human liver tissues. The relative radioactivities of the differentially protected probe DNA fragments were determined with a Fujix Bioimage Analyzer BA100 and expressed as a percentage of the total radioactivities recovered in all fragments. *A*, Variant 1 mRNA; *B*, Variant 2 mRNA; *C*, Variant 3, 4, and 5 mRNAs. The results are shown as mean values of multiple specimens with SD.

indicate that the FN gene expression is perturbed qualitatively, but not quantitatively, in liver cancer tissues.

Oncodevelopmental expression of the ED-A$^+$ and/or ED-B$^+$ FN mRNA was further demonstrated at the protein level by immunohistochemistry of HCCs with monoclonal antibodies specific to these extra peptide segments. Monoclonal antibody IST-9, specific to the ED-A segment, specifically immunostained the tumor vessels and other connective tissues of the tumor nodules, including the fibrous capsules (Fig. 6*A*). The surrounding normal tissue was negative for immunostaining. A similar staining pattern was obtained with monoclonal antibody BC-1, specific to the ED-B segment, although the intensity of the staining was less pronounced than for IST-9 (Fig. 6*B*). These results confirmed that the FN isoforms containing these extra peptide segments were synthesized and secreted by liver tumor cells and deposited at the surrounding extracellular matrix.

Alternative Splicing of Pre-mRNAs for Kininogen, Fibrinogen, and β-Amyloid Protein Precursor. We next tested whether alternative pre-mRNA splicing of other gene transcripts was similarly deregulated in malignant liver tumors. Three genes which are relatively abundantly expressed in the liver and processed by alternative RNA splicing were chosen to examine the splicing patterns in noncancerous and cancerous liver tissues.

Kininogen gene, a gene encoding the precursor protein for vasoactive kinins, generates two forms of kininogens, high-molecular-weight and low-molecular-weight kininogens, due to alternative usage of the 3′ end exons (29). In normal adult liver, mRNA for low-molecular-weight kininogen comprised about 80% of the total kininogen mRNA pool, while the mRNA for high-molecular-weight kininogen accounted for only 20% (Fig. 7). The percentage of the low-molecular-weight kininogen mRNA was essentially the same in all the tissues from chronic hepatitis, cirrhosis, and HCCs, indicating that the alternative splicing of kininogen mRNA was not affected during the course

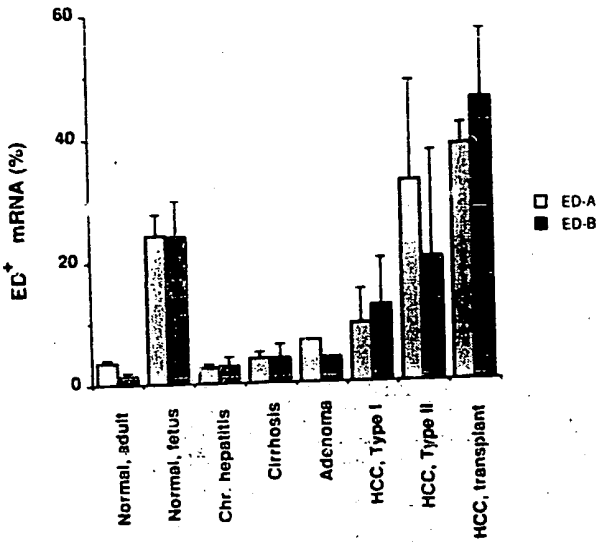

Fig. 4. Comparison of the patterns of alternative splicing of FN pre-mRNA at ED-A and ED-B regions in human liver tissues. *Ordinate*, percentage of ED-A$^+$ (□) or ED-B$^+$ (■) mRNA species within the total FN mRNA pool.

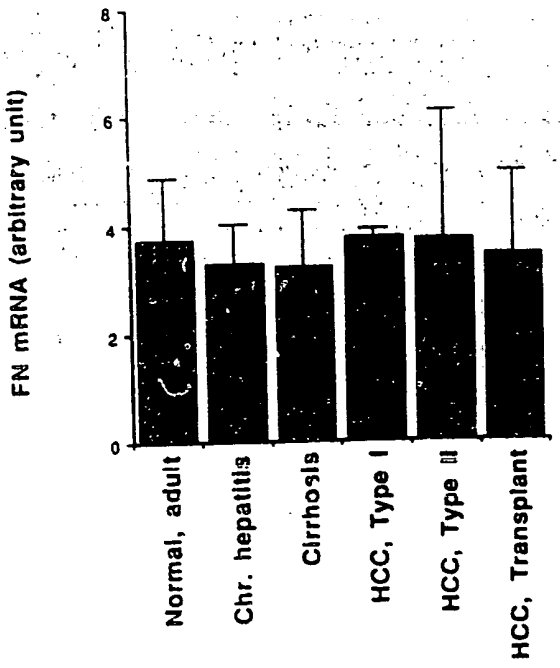

Fig. 5. Expression level of total FN mRNA in noncancerous and cancerous liver tissues. The relative abundance of total FN mRNA was determined by nuclease S1 protection analysis as described under "Materials and Methods." The levels of FN mRNA were normalized against that for β-actin mRNA.

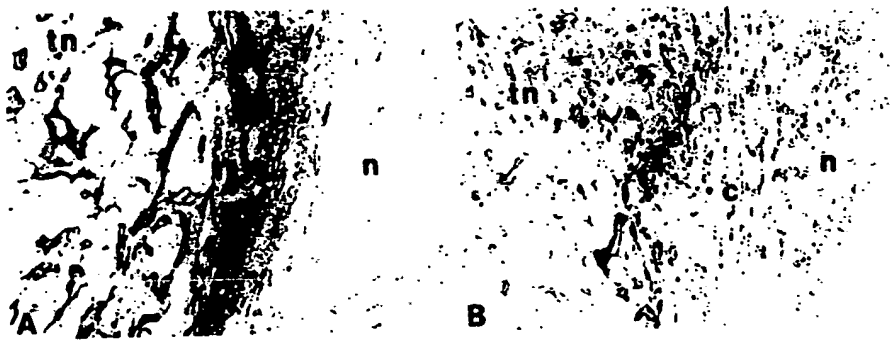

Fig. 6. Immunohistochemical localization of the ED-A$^+$ and ED-B$^+$ FN isoforms in liver tumor tissues. Tissue specimens from Type 2 HCC were immunostained with monoclonal antibodies IST-9 (*A*) and BC-1 (*B*), specific to the ED-A and ED-B peptide segments, respectively, using the avidin-biotin complex method. *m*, tumor nodule; *n*, normal liver tissue; *c*, fibrous capsule.

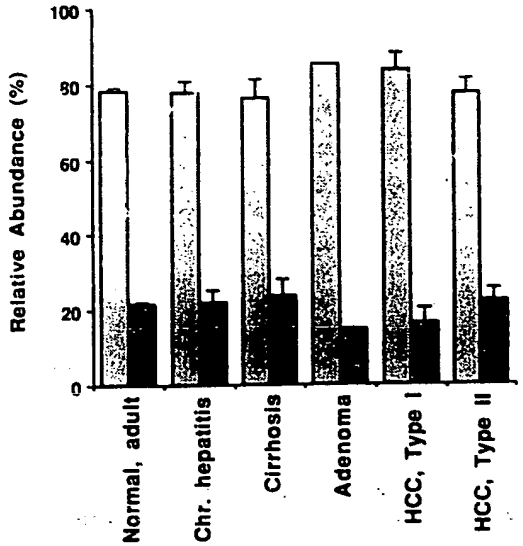

Fig. 7. Patterns of alternative splicing of human kininogen pre-mRNA in nonmalignant and malignant liver tissues. *Ordinate*, percentage of LMW (□) and HMW (■) kininogen mRNAs within the total kininogen mRNA pool.

of liver carcinogenesis. Neither form of kininogen mRNA was detectable in fetal liver by nuclease S1 protection analysis.

The gene encoding fibrinogen γ chain was also processed by alternative splicing, yielding a minor variant form γ′, which differs from γ chain in the amino acid sequence after Gln-407 (35). The percentage of the fibrinogen γ′ chain mRNA in normal adult liver was no more than 10% of the total fibrinogen mRNA pool and remained unchanged in noncancerous and cancerous liver tissues (Fig. 8). Neither form of fibrinogen mRNA was detectable in fetal liver.

The third gene we examined was the gene encoding APP. APP is a precursor protein of the β-amyloid accumulating in the brain of Alzheimer's disease patients (36) and exists as three major isoforms, APP-695, APP-751, and APP-770 (37, 38), the latter two of which contain an alternatively spliced segment homologous to Kunitz-type serine protease inhibitor. Nuclease S1 protection analysis with the antisense-stand DNA probe partially covering the exon encoding the protease inhibitor domain showed that there was no significant difference in the relative abundance of the mRNA species for APP-695, APP-751, and APP-770 among normal, noncancerous, and cancerous liver tissues (Fig. 9). The relative abundance of the three different mRNA species was essentially the same in fetal liver (data not shown). Taken together, these results suggest that the deregulation of the alternative splicing in malignant liver tumors is not ubiquitous phenomenon among all the genes being subjected to alternative RNA splicing, but rather is specific for fibronectin and some other selected genes.

DISCUSSION

Many lines of evidence indicated that tissue-specific alternative splicing of FN pre-mRNA is oncodevelopmentally regulated *in vivo* and *in vitro* (21–25, 33, 34, 39). In the liver, both the ED-A and ED-B exons are almost exclusively spliced out of the mature FN mRNA in adult tissues, although a significant percentage of the FN mRNA pool in fetal liver contains these extra exons (22, 23). Our present data clearly showed that not only the ED-A but also the ED-B exon was coordinately spliced in the FN mRNA expressed in cancerous liver tissues, indicating that the tissue-specific alternative splicing at the two ED exons was simultaneously deregulated in liver cancer. Besides the ED-A and ED-B regions, the alternative splicing at the IIICS region was also deregulated in cancerous liver tissues with a significant increase of the CS1$^+$ mRNA species. These observations provided direct evidence for the coordinate deregulation of alternative splicing of FN pre-mRNA at all three variable regions in a particular type of human cancer.

Although the physiological significance of molecular diversity of FNs at three variable regions has been only poorly understood, several lines of evidence indicated that the splicing variations at the IIICS region modulate the adhesive and migratory properties of cells (16, 19, 40). Thus, the CS1 region contains the adhesive motif EILDVPST, which selectively mediates adhesion of lymphoid cells and some tumor cells (16, 17) and promotes migration of neural crest cells *in vitro* (19). The alternative splicing at the CS1 region was also reported to modulate cell migration of aortic endothelial cells but not smooth

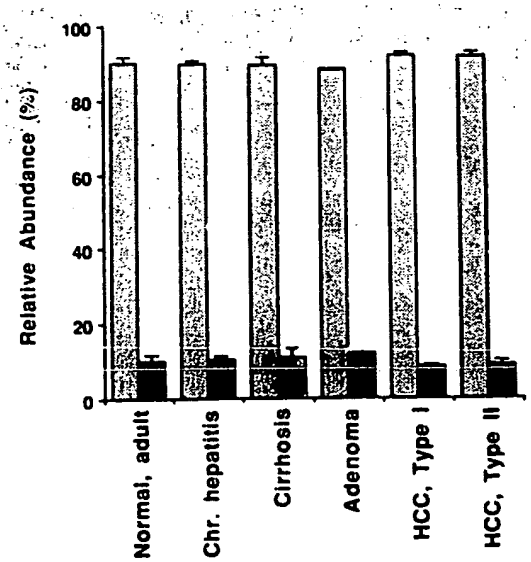

Fig. 8. Patterns of alternative splicing of human fibrinogen γ chain pre-mRNA in nonmalignant and malignant liver tissues. *Ordinate*, percentage of γ chain (□) and γ′ chain (■) mRNAs within the total fibrinogen γ chain mRNA pool.

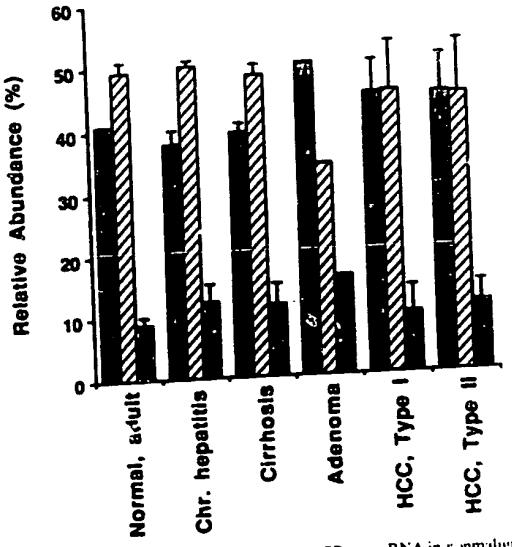

Fig. 9. Patterns of alternative splicing of human APP pre-mRNA in nonmalignant and malignant liver tissues. *Ordinate*, percentage of APP-770 (■), APP-751 (▨), and APP-695 (■) mRNAs in the total APP mRNA pool.

muscle cells (40). The relative increase of the CS1 ' mRNA species in cancerous liver tissues, therefore, may facilitate the invasion of surrounding normal tissues by tumor cells.

Because of the complexity in the splicing patterns, the IIICS region has been less extensively investigated than the ED-A and ED-B regions in its modulation of alternative splicing in human tissues and cultured cells. Hershberger and Culp (39) developed a novel double primer extension assay to quantify the relative amounts of five different mRNA variants at the IIICS region in various normal and tumor cell types. They found that the most predominant cell-type-specific differences among these cells were the abundance of the Variant 5 mRNA relative to the Variant 2 mRNA. They also showed that more than 50% of the FN mRNA pool in adult liver was Variant 5 mRNA, although the Variant 1 and 3 mRNAs were barely detectable (*i.e.*, less than 10% of the total FN pool), which was consistent with our present results. No comparison was made by these authors, however, in the splicing pattern at the IIICS region among normal, fetal, and cancerous liver tissues.

In contrast to the well-established cell adhesive function of the CS1 segment in the IIICS region, the biological significance of the presence or absence of two ED domains has not been well understood. FNs containing ED-A and/or ED-B segments are less soluble in physiological buffer solutions (41) and more readily incorporated into the extracellular matrix than FNs lacking these extra segments (42). It seems likely, therefore, that the FNs synthesized by HCCs and hence containing a significant proportion of ED-A ' and/or ED-B ' isoforms could be deposited in the extracellular matrix within the tumor nodules. In support of this notion, immunohistochemical examination of HCC tissues revealed the specific localization of ED-A '/ED-B ' FN isoforms at the tumor vessels and other connective tissues within the tumor nodules, including the fibrous capsules surrounding them. Accumulation of the ED-A '/ED-B ' isoforms, together with the increased expression of the CS1 cell adhesive site, may well be relevant to the malignant phenotypes of the tumor cells.

Although the molecular mechanism of alternative RNA splicing is largely unknown, it is generally considered that tissue-specific alternative splicing of FN pre-mRNA is regulated by nuclear *trans*-acting factor(s) which in turn may be modulated by soluble mediators such as transforming growth factor β and by cell-to-cell and/or cell-to-substrate adhesion. Transforming growth factor β has been shown to increase the relative abundance of the ED-A ' FN mRNA (43). Since the alternative splicing at three variable regions is simultaneously deregulated in cancerous liver tissues, the putative *trans*-acting factor(s) involved may well be common among the three variable regions or coregulated by another nuclear factor(s) the activities of which are perturbed in liver cancer. Identification of these *trans*-acting factor(s) as well as *cis*-acting elements flanking the alternatively spliced exons should contribute to better understanding the regulatory mechanisms of alternative splicing of FN pre-mRNA and its deregulation in malignant tumors.

Besides FN, another extracellular matrix protein tenascin has been shown to exhibit oncodevelopmental modulation of isoform diversity via alternative RNA splicing (26). Nevertheless, not all the genes processed by alternative RNA splicing are rendered for deregulated alternative splicing in malignant tumor tissues, since alternative splicing of kininogen, fibrinogen, and APP is not significantly affected in liver cancer. Our results indicated, rather, that deregulated alternative splicing of FN pre-mRNA is a specific event during the course of liver carcinogenesis and raise the possibility that FN isoforms containing three alternatively spliced extra segments may play an important role in the expression of invasive phenotypes by malignant tumor cells.

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REFERENCES

1. Petersen, T. E., Skorstengaard, K., and Vibe-Pedersen, K. Primary structure of fibronectin. *In:* D. F. Mosher (ed.), Fibronectin, pp. 1–24. San Diego: Academic Press, 1989.
2. Ruoslahti, E. Fibronectin and its receptors. Annu. Rev. Biochem., *57:* 375–413, 1988.
3. Yamada, K. M. Fibronectin domains and receptors. *In:* D. F. Mosher (ed.), Fibronectin, pp. 47–121. San Diego: Academic Press, 1989.
4. Yamada, K. M. Fibronectin and other cell interactive glycoproteins. *In:* E. D. Hay (ed.), Cell Biology of Extracellular Matrix, pp. 111–146. New York: Plenum Press, 1991.
5. Yamada, K. M., and Kennedy, D. W. Fibroblast cellular and plasma fibronectins are similar but not identical. J. Cell Biol., *80:* 492–498, 1979.
6. Sekiguchi, K., Siri, A., Zardi, L., and Hakomori, S. Differences in domain structure between human fibronectins isolated from plasma and from culture supernatants of normal and transformed fibroblasts. J. Biol. Chem., *260:* 5105–5114, 1985.
7. Schwarzbauer, J. E., Tamkun, J. W., Lemischka, I. R., and Hynes, R. O. Three different fibronectin mRNAs arise by alternative splicing within the coding region. Cell, *35:* 421–431, 1983.
8. Kornblihtt, A. R., Vibe-Pedersen, K., and Baralle, F. E. Human fibronectin: cell specific alternative mRNA splicing generates polypeptide chains differing in the number of internal repeats. Nucleic Acids Res., *12:* 5853–5868, 1984.
9. Sekiguchi, K., Klos, A. M., Kurachi, K., Yoshitake, S., and Hakomori, S. Human liver fibronectin complementary DNAs: identification of two different messenger RNAs possibly encoding the α and β subunits of plasma fibronectin. Biochemistry, *25:* 4936–4941, 1986.
10. Zardi, L., Carnemolla, B., Siri, A., Petersen, T. E., Paolella, G., Sebastio, G., and Baralle, F. E. Transformed human cells produce a new fibronectin isoform by preferential alternative splicing of a previously unobserved exon. EMBO J., *6:* 2337–2342, 1987.
11. Gutman, A., and Kornblihtt, A. R. Identification of a third region of cell-specific alternative splicing in human fibronectin mRNA. Proc. Natl. Acad. Sci. USA, *84:* 7179–7182, 1987.
12. Vibe-Pedersen, K., Kornblihtt, A. R., and Petersen, T. E. Expression of a human α-globin/fibronectin gene hybrid generates two mRNAs by alternative splicing. EMBO J., *3:* 2511–2516, 1984.
13. Schwarzbauer, J. E., Patel, R. S., Fonda, D., and Hynes, R. O. Multiple sites of alternative splicing of the rat fibronectin gene transcript. EMBO J., *6:* 2573–2580, 1987.
14. Tamkun, J. W., Schwarzbauer, J. E., and Hynes, R. O. A single rat fibronectin gene generates three different mRNAs by alternative splicing of a complex exon. Proc. Natl. Acad. Sci. USA, *81:* 5140–5144, 1984.
15. Bernard, M. P., Kolbe, M., Weil, D., and Chu, M. L. Human cellular fibronectin: comparison of the C-terminal portion with rat identifies primary structural domains separated by hypervariable regions. Biochemistry, *24:* 2698–2704, 1985.
16. Humphries, M. E., Komoriya, A., Akiyama, S. K., Olden, K., and Yamada, K. M.

Identification of two distinct regions of the type III connecting segment of human plasma fibronectin that promote cell type-specific adhesion. J. Biol. Chem., *262:* 6886–6892, 1987.

17. Wayner, E. A., Garcia-Pardo, A., Humphries, M. J., McDonald, J. A., and Carter, W. G. Identification and characterization of the T lymphocyte adhesion receptor for an alternative cell attachment domain (CS-1) in plasma fibronectin. J. Cell Biol., *109:* 1321–1330, 1989.
18. Guan, J-L., and Hynes, R. O. Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor $\alpha_4\beta_1$. Cell, *60:* 53–61, 1990.
19. Dufour, S., Duband, J-L., Humphries, M. J., Obara, M., Yamada, K. M., and Thiery, J. P. Attachment, spreading and locomotion of avian neural crest cells are mediated by multiple adhesion sites on fibronectin molecules. EMBO J., *7:* 2661–2671, 1988.
20. Saiki, I., Murata, J., Makabe, T., Matsumoto, Y., Ohdate, Y., Kawase, Y., Taguchi, Y., Shimojo, T., Kimizuka, F., Kato, I., and Azuma, I. Inhibition of lung metastasis by synthetic and recombinant fragments of human fibronectin with functional domains. Jpn. J. Cancer Res., *81:* 1003–1011, 1990.
21. Ffrench-Constant, C., and Hynes, R. O. Alternative splicing of fibronectin is temporally and spatially regulated in the chicken embryo. Development (Camb.), *109:* 375–388, 1989.
22. Oyama, F., Murata, Y., Suganuma, N., Kimura, T., Titani, K., and Sekiguchi, K. Patterns of alternative splicing of fibronectin pre-mRNA in human adult and fetal tissues. Biochemistry, *28:* 1428–1434, 1989.
23. Pagani, F., Zagato, L., Vergani, C., Casari, G., Sidoli, A., and Baralle, F. E. Tissue-specific splicing pattern of fibronectin messenger RNA precursor during development and aging in rat. J. Cell Biol., *113:* 1223–1229, 1991.
24. Oyama, F., Hirohashi, S., Shimosato, Y., Titani, K., and Sekiguchi, K. Deregulation of alternative splicing of fibronectin pre-mRNA in malignant human liver tumors. J. Biol. Chem., *264:* 10331–10334, 1989.
25. Oyama, F., Hirohashi, S., Shimosato, Y., Titani, K., and Sekiguchi, K. Oncodevelopmental regulation of the alternative splicing of fibronectin pre-messenger RNA in human lung tissues. Cancer Res., *50:* 1075–1078, 1990.
26. Oyama, F., Hirohashi, S., Shimosato, Y., Titani, K., and Sekiguchi, K. Qualitative and quantitative changes of human tenascin expression in transformed lung fibroblast and lung tumor tissues: comparison with fibronectin. Cancer Res., *51:* 4876–4881, 1991.
27. Kanai, T., Hirohashi, S., Upton, M. P., Noguchi, M., Kishi, K., Makuuchi, M., Yamasaki, S., Hasegawa, H., Takayasu, K., Moriyama, N., and Shimosato, Y. Pathology of small hepatocellular carcinoma: a proposal for a new gross classification. Cancer (Phila.), *60:* 810–819, 1987.
28. Chomczynski, P., and Sacchi, N. Single-step method of RNA isolation by guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem., *102:* 156–159, 1987.
29. Ohkubo, I., Kurachi, K., Takawawa, T., Shikawa, H., and Sasaki, M. Isolation of a human cDNA for α_2-thiol proteinase inhibitor and its identity with low molecular weight kininogen. Biochemistry, *23:* 5691–5697, 1984.
30. Chung, D. W., Chan, W-Y., and Davie, E. W. Characterization of a complementary deoxyribonucleic acid coding for the γ chain of human fibrinogen. Biochemistry, *22:* 3250–3256, 1983.
31. Oyama, F., Shimada, H., Oyama, R., Titani, K., and Ihara, Y. Differential expression of β amyloid protein precursor (APP) and tau mRNA in the aged human brain: individual variability and correlation between APP-751 and four-repeat tau. J. Neuropathol. Exp. Neurol., *50:* 560–578, 1991.
32. Berk, A., and Sharp, P. A. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell, *12:* 721–732, 1977.
33. Borsi, L., Carnemolla, B., Castellani, P., Rosellini, C., Vecchio, D., Allemanni, G., Chang, S. E., Taylor-Papadimitriou, J., Pande, H., and Zardi, L. Monoclonal antibodies in the analysis of fibronectin isoforms generated by alternative splicing of mRNA precursors in normal and transformed human cells. J. Cell Biol., *104:* 595–600, 1987.
34. Carnemolla, B., Balza, E., Siri, A., Zardi, L., Nicotra, M. R., Bigotti, A., and Natali, P. G. A tumor-associated fibronectin isoform generated by alternative splicing of messenger RNA precursors. J. Cell Biol., *108:* 1139–1148, 1989.
35. Chung, D. W., and Davie, E. W. γ and γ′ chains of human fibrinogen are produced by alternative mRNA processing. Biochemistry, *23:* 4232–4236, 1984.
36. Kang, J., Lemaire, H-G., Unterbeck, A., Salbaum, J. M., Masters, C. L., Gezeschick, K-H., Multhaup, G., Beyreuther, K., and Muller-Hill, B. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. Nature (Lond.), *325:* 733–736, 1987.
37. Ponte, P., Gonzalez-DeWhitt, P., Schilling, J., Miller, J., Hsu, D., Greenberg, B., Davis, K., Wallace, W., Lieberburg, I., Fuller, F., and Cordell, B. A new A4 amyloid mRNA contains a domain homologous to serine proteinase inhibitors. Nature (Lond.), *331:* 525–527, 1988.
38. Kitaguchi, N., Takahashi, Y., Tokushima, Y., Shiojiri, S., and Ito, H. Novel precursor of Alzheimer's disease protease inhibitory activity. Nature (Lond.), *331:* 530–532, 1988.
39. Hershberger, R. P., and Culp, L. A. Cell-type-specific expression of alternatively spliced human fibronectin IIICS mRNAs. Mol. Cell. Biol., *10:* 662–671, 1990.
40. Kocher, O., Kennedy, S. P., and Madri, J. A. Alternative splicing of endothelial cell fibronectin mRNA in the IIICS region. Am. J. Pathol., *137:* 1509–1524, 1990.
41. Yamada, K. M., Schlessinger, D. H., Kennedy, D. W., and Pastan, I. Characterization of a major fibroblast cell surface glycoprotein. Biochemistry, *16:* 5552–5559, 1977.
42. Guan, J-L., Trevithick, J. E., and Hynes, R. O. Retroviral expression of alternatively spliced forms of rat fibronectin. J. Cell Biol., *110:* 833–847, 1990.
43. Balza, E., Borsi, L., Allemanni, G., and Zardi, L. Transforming growth factor β regulates the levels of different fibronectin isoforms in normal human cultured fibroblasts. FEBS Lett., *228:* 42–44, 1988.

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Thorpe et al., "Selective Killing of Proliferating Vascular Endothelial Cells by an Anti-Fibronectin Receptor Immunotoxin," 16th LH Gray Conference, University of Manchester Institute of Science and Technology, Sep. 17-21, 1990.

Other Reference Publication (89):
Aoyagi, "Distribution of Plasma Fibronectin in the Metastatic Lesion of Cancer: Experimental Study by Autoradiography," Thrombosis Research, 49:265-75, 1988.

Other Reference Publication (96):
Burton-Wurster, et al., "Expression of the Ed B Fibronectin Isoform in Adult Human Articular Cartilage," Biochemical and Biophysical Research Communication 165(2): 782-87, 1989.

Other Reference Publication (98):
Carnemolla et al., "A Tumor-associated Fibronectin Isoform Generated by Alternative Splicing of Messenger RNA Precursors," The Journal of Cell Biology, 108:1139-48, 1989.

Other Reference Publication (99):
Carnemolla et al., "The Inclusion of the Type III Repeat ED-B in the Fibronectin Molecule Generates Conformational Modifications that Unmask a Cryptic Sequence," The Journal of Biological Chemistry, 267(34):245898-92, 1992.

Other Reference Publication (100):
Carnemolla, et al., "Phage Antibodies with Pan-Species Recognition of the Oncofoetal Angiogenesis Marker Fibronectin ED-B Domain," Int. J. Cancer 68:397-405, 1996.

Other Reference Publication (101):
Castellani, et al., "The Fibronectin Isoform Containing the ED-B Oncofoetal Domain: A Marker of Angiogenesis," Int. J. Cancer, 59:612-18, 1994.

Other Reference Publication (104):
Farnoud, et al., "Fibronectin Isoforms Are Differentially Expressed in Normal and Adenomatous Human Anterior Pituitaries," Int. J. Cancer, 61:27-34, 1995.

Other Reference Publication (108):
Kaczmarek, et al., "Distribution of Oncofetal Fibronectin Isoforms in Normal Hyperplastic and Neoplastic Human Breast Tissues," Int. J. Cancer, 58:11-16, 1994.

Other Reference Publication (112):
Linnala, et al., "Human Amnion Epithelial Cells Assemble Tenascins and Three Fibronectin Isoforms in the Extracellular Matrix," FEBS, 314(1,2):74-78, 1993.

Other Reference Publication (115):
Oyama, et al., "Coordinate Oncodevelopmental Modulation of Alternative Splicing of Fibronectin Pre-Messenger RNA at ED-A, ED-B and CS1 Regions in Human Liver Tumors," Cancer Research 53:2005-11, 1993.

Other Reference Publication (116):
Peters, et al., "Expression of the Alternatively Spliced EIIB Segment of Fibronectin," Cell Adhesion and Communication, 3:67-89, 1995.

Other Reference Publication (126):
Saiki et al., "Inhibition of Tumor Angiogenesis by a Synthetic Cell-Adhesive Polypeptide Containing the Arg-Gly-Asp (RGD) Sequence of Fibronectin, Poly(RGD)," Jpn. J. Cancer Res. 81:668-75, 1990.

Other Reference Publication (128):
Schwarzbauer, "Alternative Splicing of Fibronectin: Three Variants, Three Functions," BioEssays 13(10)527-33, 1991.

Other Reference Publication (133):
Ueda, et al., "Selective Distribution of Fibronectin to a Tumor-Cell Line," Cancer Letters 31:261-65, 1986.

Other Reference Publication (134):
Vartio, et al, "Differential Expression of the ED Sequence-Containing Form of Cellular Fibronectin in Embryonic and Adult Human Tissues," Journal of Cell Science, 88:419-30, 1987.

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PHAGE ANTIBODIES WITH PAN-SPECIES RECOGNITION OF THE ONCOFOETAL ANGIOGENESIS MARKER FIBRONECTIN ED-B DOMAIN

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Fibronectin (FN) exists in several polymorphic forms due to alternative splicing. The B-FN isoform (with ED-B domain inserted by splicing) is present in the stroma of foetal and neoplastic tissues and in adult and neoplastic blood vessels during angiogenesis but is undetectable in mature vessels. This isoform, therefore, represents a promising marker for angiogenesis, as already shown using the mouse monoclonal antibody (MAb) BC-1 directed against an epitope on human B-FN. However, this MAb does not directly recognise the human ED-B domain nor does it recognise B-FN of other species; therefore, it cannot be used as a marker of angiogenesis in animal models. In principle, antibodies directed against the human ED-B domain should provide pan-species markers for angiogenesis as the sequence of this domain is highly conserved in different species (and identical in humans and mice). As it has proved difficult to obtain such antibodies by hybridoma technology, we used phage display technology. Here, we describe the isolation of human antibody fragments against the human ED-B domain that bind to human, mouse and chicken B-FN. As shown by immunohistochemistry, the antibody fragments stain human neoplastic tissues and the human, mouse and chicken neovasculature.

During tumour progression, the extracellular matrix of the tissues in which a tumour grows is remodelled through proteolytic degradation of the extracellular matrix components and by neosynthesis of extracellular matrix components (by both neoplastic cells and stromal cells). This generates a "tumoral extracellular matrix" which differs from that of normal tissues. It appears that this tumoral extracellular matrix generates a more suitable environment for tumour progression (inductive and/or instructive), of which angiogenesis is a crucial step (Folkman, 1995; Van den Hoff, 1988; Risau and Lemmon, 1988).

The tumoral extracellular matrix contains several "tumour-associated" antigens that are, in general, more abundant and possibly more stable than "tumour-associated" antigens of the cell surface. Among these components are the large isoforms generated by a modified pattern of alternative splicing of the mRNA precursors of tenascin and fibronectin (FN) (Borsi *et al.*, 1992*b*, Carnemolla *et al.*, 1989; Castellani *et al.*, 1994; Kaczmarek *et al.*, 1994; Leprini *et al.*, 1994).

FNs are high-molecular-mass adhesive glycoproteins present in the extracellular matrix and body fluids. These molecules are involved in different biological processes, such as the establishment and maintenance of normal cell morphology, cell migration, haemostasis and thrombosis, wound healing and oncogenic transformation (Hynes, 1990 and references therein). FN polymorphism is due to alternative splicing patterns in 3 regions (IIICS, ED-A and ED-B) of the single FN primary transcript as well as to post-translational modifications. In transformed cells and in malignancies, the splicing pattern of FN-pre-mRNA is altered (Castellani *et al.*, 1986, 1994; Borsi *et al.*, 1987, 1992*a*; Vartio *et al.*, 1987; Zardi *et al.*, 1987; Carnemolla *et al.*, 1989; Oyama *et al.*, 1989; Kaczmarek *et al.*, 1994), leading to an increased expression of FN isoforms containing the IIICS, ED-A and ED-B sequences.

In particular, the FN isoform containing the ED-B sequence (B-FN) (Zardi *et al.*, 1987), which, with some very rare exceptions, is undetectable in normal adult tissues, exhibits a much greater expression in foetal and tumour tissues as well as during wound healing (Zardi *et al.*, 1987; Norton and Hynes, 1987; Carnemolla *et al.*, 1989; french-Constant *et al.*, 1989). Furthermore, B-FN is accumulated around neovasculature during angiogenetic processes (Castellani *et al.*, 1994) and thereby provides a marker for angiogenesis.

One monoclonal antibody (mAb), BC-1, has been isolated against the human B-FN isoform (Carnemolla *et al.*, 1989) and has been used widely to study angiogenesis. However, this antibody does not recognise the "inserted" ED-B domain directly; it appears to recognise an epitope within the type III repeat 7, which precedes the ED-B (Fig. 1). This epitope is cryptic in FN molecules lacking ED-B and unmasked in molecules containing this domain (Carnemolla *et al.*, 1992); indeed, it is conceivable that the cryptic epitope in FN could be unmasked in other circumstances. Furthermore, the antibody BC-1 is highly specific to human B-FN and does not bind mouse or chicken B-FN; therefore, it cannot be used to follow angiogenesis in animal models.

As the sequence of the ED-B domain is highly conserved in different species (and identical in humans and mice), antibodies directed to the ED-B domain should provide a pan-species marker for angiogenesis. Perhaps due to the conservation of sequence, it has proved difficult to obtain such antibodies by hybridoma technology. Rabbit polyclonal antibodies to the ED-B sequence have been produced (Peters *et al.*, 1995). However, in addition to the intrinsic limitations of polyclonal antibodies, these reagents require treatment of FN or tissues with N-glycanase.

Here, we explored the use of phage display technology (Smith, 1985; McCafferty *et al.*, 1990; for review see Winter *et al.*, 1994) to isolate human antibody fragments binding directly to the human ED-B domain. Repertories of single-chain Fv fragments (Huston *et al.*, 1988; Bird *et al.*, 1988) derived from human V-gene segments (Nissim *et al.*, 1994) were displayed on phage and selected using the immobilised recombinant human ED-B domain. The antibody fragments secreted into bacterial cultures (Skerra and Pluckthun, 1988; Better *et al.*, 1988; Glockshuber *et al.*, 1991) were characterised by binding to FN isoforms and fragments and by immunohistochemistry.

MATERIAL AND METHODS

Purification of plasma and cellular FN, thermolysin digestion of FNs and purification of tenascin-C

FN was purified from human plasma and from conditioned media of the WI38VA13 cell line as previously reported (Zardi *et al.*, 1987). Purified FNs were digested with thermolysin (Protease type X; Sigma, St. Louis, MO) as reported by Carnemolla *et al.*, (1989). Native FN-110 kDa (B^-) and native

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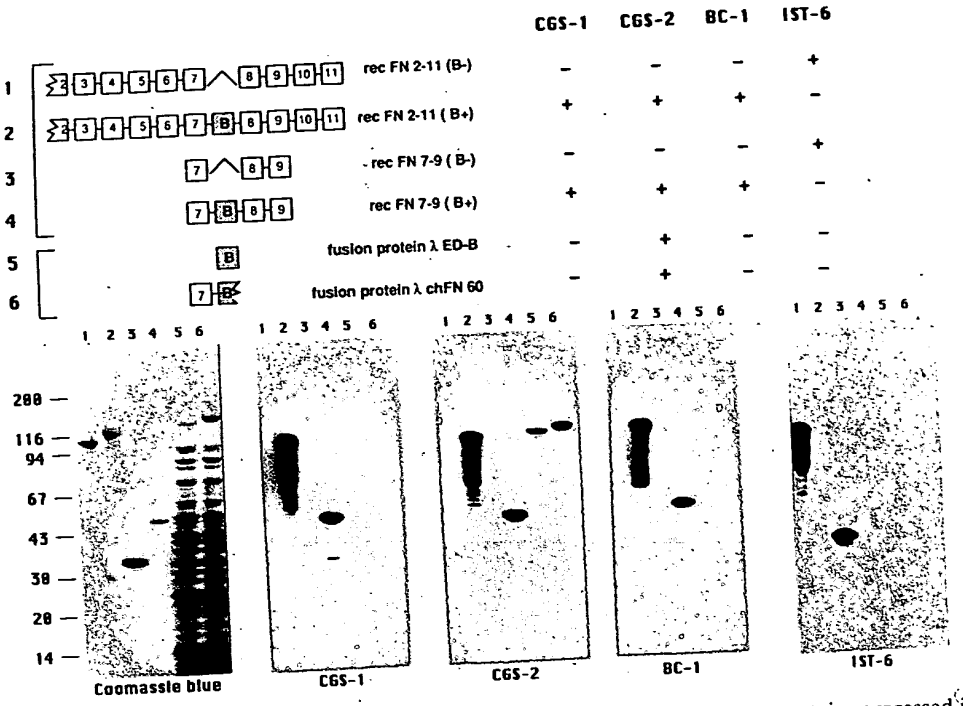

FIGURE 1 – Above, the FN type III repeat sequences contained in the fusion and recombinant proteins expressed in *E. coli* and the reactivity of these proteins with CGS-1 and -2 and with MAbs BC-1 and IST-6. Below, Coomassie blue staining and immunoblots with CGS-1, -2, BC-1 and IST-6 of the peptides depicted above. Lanes 1–6 correspond to the number of the various recombinant and fusion proteins shown above. Values on the left indicate molecular masses (in kilodaltons) of the standards.

FN-120 kDa (B^+) fragments (see Fig. 1) were purified from FN-thermolysin digests as previously reported (Carnemolla *et al.*, 1989). SDS-PAGE and immunoblotting were carried out as described by Carnemolla *et al.*, (1989).

FN-recombinant fragments and fusion proteins

For recombinant FN peptides containing the type III repeats 2-11(B^-) and 2-11 (B^+) (Fig. 2), we made a construct using FN cDNA from the clones pFH154 (Kornblihtt *et al.*, 1985) λF10 and λF2 (Carnemolla *et al.*, 1989). The cDNA constructs, which included the bases from 2229 to 4787 (Kornblihtt *et al.*, 1985), were inserted into pQE-3/5 vectors using the QIAexpress kit (Qiagen, Chatsworth, CA). The recombinants FN-III 2-11 (B^-) and (B^+) were purified by immunoaffinity chromatography as previously reported (Carnemolla *et al.*, 1989).

Recombinant FN fragments containing the type III homology repeats 7B89, 789, ED-B and 6 were produced by PCR amplification using UlTma DNA-polymerase (Perkin Elmer, Norwalk, CT), and cDNA from clones FN2-11 (B^+) and FN2-11 (B^-) with appropriated primers. PCR products were cloned into pQE-12 vector using the QIAexpress kit and expressed in *Escherichia coli.* All cloned cDNAs were sequenced using a Sequenase 2.0 DNA sequencing kit (USB, Cleveland, OH). Recombinant proteins were purified by Ni-NTA columns (Qiagen) by use of the His6 tag appended at the C-terminus of these FN fragments. All procedures were carried out according to manufacturer's instructions. The ED-B-βGal fusion protein was prepared by cloning the ED-B cDNA into λgt11 phage. The clone pchfn60 (Norton and Hynes, 1987), used to prepare the clone λchFN60 producing a fusion protein containing part of the ED-B sequence, was a generous gift of Dr. R.O. Hynes (Center for Cancer Research, M.I.T., Cambridge, MA). For the immunoblotting analysis, FN fusion proteins were prepared as described by Carnemolla *et al.* (1989).

Antibody fragment isolation

A human scFv phage library (Nissim *et al.*, 1994) was used for the selection of recombinant antibodies. We used 2 different antigens for the selection, and in each case 3 rounds of panning were performed. The first antigen was a recombinant FN fragment containing the complete type III repeats 7B89. The antigen was coated overnight at 4°C on immunotubes (MaxiSorp; Nunc, Roskilde, Denmark) at a concentration of 50 μg/ml in PBS (20 mM phosphate buffer, 0.15 M NaCl, pH 7.2). The second antigen used was the recombinant ED-B peptide (Zardi *et al.*, 1987) that was covalently immobilised on ELISA wells (Covalink; Nunc) at 50 μg/ml by overnight incubation at room temperature. At the end of the third round of panning, phages eluted from the immunoabsorbent were used to infect HB2151 *E. coli* cells and plated as described (Nissim *et al.*, 1994). In each selection, 95 ampicillin-resistant single colonies were screened to identify by ELISA those producing antigen-binding scFv fragments (Nissim *et al.*, 1994). The clones which gave the strongest ELISA signals were selected for further analysis by immunohistochemistry on frozen human tumour sections and by immunoblot on different ED-B-containing FN fragments.

Two clones were subjected to a process of affinity maturation (using *in vitro* mutagenesis and further rounds of phage selection; data not shown). The resulting matured clones CGS-1 (from 7B89) and CGS-2 (from ED-B) were selected and subcloned in the Sfi1/Not1 sites of the pDN268 expression vector (Neri *et al.*, 1996), which appends a phosphorylable tag, the FLAG epitope and a His_6 tag at the C-terminal extremity of the scFv.

Antibody purification

Single bacterial colonies were grown at 37°C in 2 × TY medium containing 100 μg/l ampicillin and 0.1% glucose. When the cell suspension reached OD_{600} = 0.8, isopropyl-β-D-

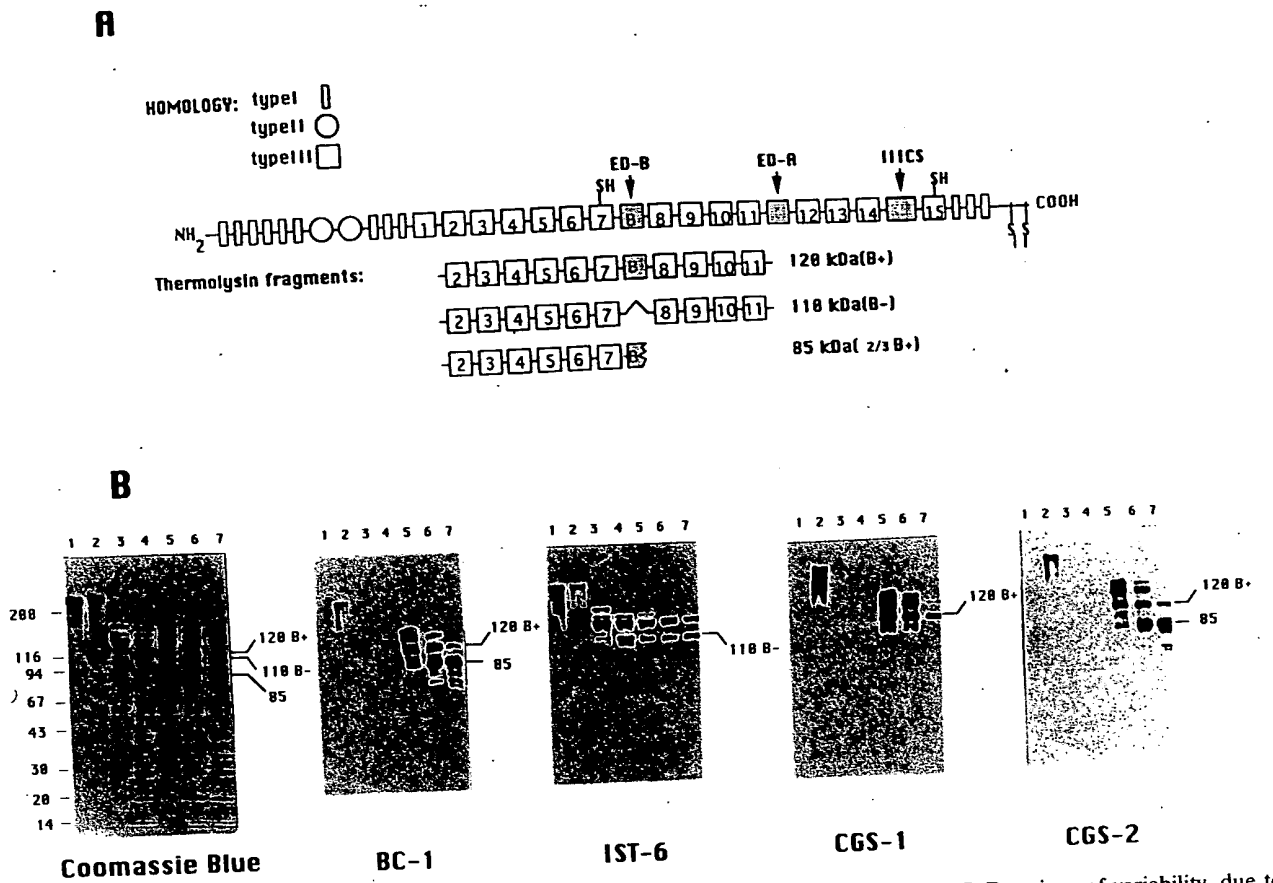

FIGURE 2 – *(a)* Model of the domain structure of a human FN subunit. The IIICS, ED-A and ED-B regions of variability, due to alternative splicing of the FN pre-mRNA, are indicated. The figure also indicates the internal homologies as well as the main thermolysin fragments containing ED-B (Zardi *et al.*, 1987). *(b)* SDS-PAGE (4–18%) of plasma and WI38VA FN and their thermolysin digests stained with Coomassie blue and immunoblots stained with BC-1, IST-6, CGS-1 and -2. Undigested (lane 1) and digested plasma FN using 2 concentrations of thermolysin (1 μg/mg of FN, lane 3, and 10 μg/mg of FN, lane 4). Undigested (lane 2) and digested WI38VA13 FN using 3 concentrations of thermolysin (1 μg/mg of FN, lane 5; 5 μg/mg, lane 6; and 10 μg/mg, lane 7). Numbers on the right indicate the main thermolysin fragments shown in *(a)*. Values on the left indicate molecular masses (in kilodaltons) of the standards.

thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and growth continued for 16–20 hr at 30°C. After centrifugation (4,000*g*, 30 min), the supernatant was filtered, concentrated and exchanged into loading buffer (50 mM phosphate, pH 7.4, 500 mM NaCl, 20 mM imidazole) using a Minisette (Filtron, Karlstein, Germany) tangential flow apparatus. The resulting solution was loaded onto 1 ml Ni-NTA resin (Qiagen), washed with 50 ml loading buffer and eluted with elution buffer (loading buffer + 100 mM imidazole, pH 7.4). The purified antibody was checked by SDS-PAGE and dialysed *vs.* PBS at 4°C. Alternatively scFv(s) were purified by immunoaffinity using the recombinant FN fragment 7B89 conjugated to Sepharose 4B (Pharmacia, Uppsala, Sweden). Binding affinities of the phage antibodies were measured by plasmon resonance.

ELISA immunoassay

Purified FN from plasma or the WI38VA cell line, native or recombinant FN fragments and tenascin in PBS at a concentration of 50–100 μg/ml were incubated in wells of Immuno-Plate (Nunc) overnight at 4°C. Antigens were removed and wells washed with PBS, saturated with PBS containing 3% BSA and incubated 2 hr at 37°C. After 4 washes with PBS containing Tween 20 at a final concentration of 0.05% (PBST), wells were incubated with the MAbs BC-1 and IST-6 or a fresh mixture of scFv and antibodies to the tag peptide [the MAb M2 (Kodak, New Haven, CT) for the FLAG tag or the MAb 9E10 (ATCC, Rockville, MD) for the Myc tag] at 37°C for 90 min, washed 4 times with PBST and incubated with biotinylated goat anti-mouse IgG (Bio-SPA, Milan, Italy) diluted 1/2,000 in 3% BSA in PBST for 1 hr at 37°C. After 4 washes with PBST, streptavidin-biotinylated alkaline phosphatase complex (Bio-SPA) diluted 1/800 in PBST containing 2 mM $MgCl_2$, was added and the plate incubated 1 hr at 37°C. The reaction was developed using phosphatase substrate tablets (Sigma) in diethanolamine 10%, pH 9.8, and the optical density measured at 405 nm.

Tissues, immunohistochemical procedures and MAbs

Normal and neoplastic tissues were obtained from samples taken during the course of therapeutic surgical procedures. For immunohistochemical studies, 5-μm-thick cryostat sections were air-dried and fixed in cold acetone for 10 min. Immunostaining was performed using a streptavidin-biotin alkaline phosphatase complex staining kit (Bio-Spa) and naphtol-AS-MX-phosphate and fast-red TR (Sigma). Gill's haematoxylin was used as a counter-stain, followed by mounting in glycergel (Dako, Carpenteria, CA) as previously reported (Castellani *et al.*, 1994). The MAbs used were BC-1, which recognises the B-FN isoform; IST-6, which recognises

only FN molecules without the ED-B sequence; and IST-4, which is specific for all FN isoforms. The characterisation of these antibodies has been previously reported (Carnemolla *et al.*, 1989, 1992). Six-week-old SCID mice were injected s.c. with 10^6 cells of the murine teratocarcinoma F9 (ATCC). Animals were killed when tumours reached a diameter of 1–1.5 cm. SCID mice were used to avoid background in immunohistochemical experiments due to endogenous immunoglobulins.

RESULTS

Isolation of 2 human antibody fragments against the ED-B domain

The phage antibody library (Nissim *et al.*, 1994) was selected using recombinant FN fragments corresponding to the type III homology repeats 7B89 and ED-B (see "Material and Methods"). After 3 rounds of panning of the phage, secreted scFv fragments from bacterial cultures were screened for binding to the ED-B domain by ELISA. Several clones were identified with good ELISA signals, which also stained glioblastoma multiforme and breast cancer sections.

Two phage antibody clones, selected with the 7B89 and the ED-B FN recombinant fragments, were then subjected to a process of affinity maturation *in vitro*. This yielded clones CGS-1 and CGS-2, respectively, with binding affinities to the ED-B domain of 53 nM and 1.1 nM, respectively. Sequencing of the V-genes of CGS-1 and CGS-2, identified human V_H segment DP47 and V_λ segment DPL-16, with VH-CDR3 sequences of SLPK and GVGAFRPYRKHE and V_λ-CDR3 sequences of NSS-PVVLNG-VV and NSS-PFEHNL-VV, respectively (Williams and Winter, 1993).

CGS-1 and CGS-2 are directed to different epitopes within the ED-B domain and specifically recognise whole native B-FN molecules

The binding of CGS-1 and -2 to human FN fragments and isoforms and to recombinant FN fragments was analysed by ELISA (Table I). CGS-1 and -2 recognised the recombinant ED-B domain as well as all natural or recombinant FN fragments containing this domain but did not bind to FN fragments lacking the domain (or to tenascin which contains 15 type III homology repeats). CGS-1 and -2 did not bind to plasma FN, which mostly lacks the ED-B domain (Zardi *et al.*, 1987), but did bind to FN from the SV40-transformed cell line WI38VA, which mostly contains B-FN (Zardi *et al.*, 1987; Borsi *et al.*, 1992*a*). Binding of CGS-1 and -2 to B-FN was inhibited by the recombinant ED-B domain (not shown). Binding of CGS-1 and -2 to recombinant human FN fragments and fusion proteins was also analysed by immunoblotting (Fig. 1). As expected, the results were consistent with the ELISA data.

TABLE I – IMMUNOREACTION OF scFv OR MAbs WITH DIFFERENT ANTIGENS[1]

	CGS-1	CGS-2	BC-1	IST-6
Plasma FN	0.07	0.04	0.09	1.73
WI38-VA FN	1.16	0.72	1.20	1.12
n110 kDa (B^-)	0.03	0.01	0.05	1.20
n120 kDa (B^+)	0.82	0.81	1.20	0.02
rec FN7B89	1.11	1.02	1.02	0.01
rec FN789	0.01	0.01	0.05	1.25
rec EDB	1.21	1.32	0.15	0.04
rec FN6	0.01	0.01	0.08	0.03
Tenascin	0.01	0.02	0.06	0.02

[1]The values represent the OD at 405 nm in ELISA after background subtraction as reported in "Material and Methods" and are the mean of 4 replicate experiments which showed no more than 10% difference.

Clearly, CGS-1 and -2 recognise the ED-B domain, whether isolated or as part of FN. Indeed, the binding affinities of CGS-1 and -2 to B-FN from WI38VA as measured by surface plasmon resonance (12 and 2.4 nM, respectively) were similar to those of the isolated ED-B domain. This contrasts with MAb BC-1, which recognises the B-FN isoform (but not the ED-B domain), and with MAb IST-6, which recognises only FN lacking the ED-B domain (Carnemolla *et al.*, 1992).

Immunoblots were also performed with FN from plasma and WI38VA cells and with their thermolysin digests (Fig. 2). Upon thermolysin digestion, FN from WI38VA cells (mostly containing ED-B) generates a major 120-kDa fragment (repeats 2–11 and containing the ED-B domain) and a minor 110-kDa fragment lacking the ED-B domain (Fig. 2). Further digestion of the 120-kDa domain generates 2 fragments: an 85-kDa fragment (repeats 2–7 and the N-terminal portion of ED-B comprising most of this domain; Zardi *et al.*, 1987) and a 35-kDa fragment (Zardi *et al.*, 1987). Immunoblots indicate that CGS-1 and -2 did not bind to plasma FN or its digests but did bind to ED-B-rich FN from WI38VA cells and its digests. This provides further confirmation of the binding of CGS-1 and -2 to the ED-B domain. However, whereas CGS-1 bound to the 120-kDa fragment but not the 85-kDa fragment, CGS-2 bound to both fragments (Fig. 2), suggesting that CGS-1 and -2 recognise different epitopes within the ED-B sequence.

Antibody fragments CGS-1 and -2 recognise human neoplastic tissues and neovasculature

CGS-1 and -2 were used to immunolocalise B-FN in various human normal and neoplastic tissues, and the pattern of staining was compared with that of the MAb BC-1 and with those of other MAbs able to recognise all of the FN isoforms (IST-4) or the ED-B-lacking FN isoform (IST-6). The results showed that both CGS-1 and -2 reacted with the same histological structures recognised by the MAb BC-1. Indeed, we observed negative reactions with a number of normal human tissues and positive reactions with neoplastic tissues. Figure 3 shows that in normal human skin, while a large amount of ED-B-lacking FN is present in the derma, neither the MAb BC-1 nor CGS-1 and -2 showed positive reaction.

Using MAb BC-1, we have previously shown in invasive ductal breast cancers, that B-FN, while having a more restricted distribution with respect to total FN, was detectable in more than 95% of cases (Kaczmarek *et al.*, 1994). As shown in Figure 4, total FN has a homogeneous distribution within the stroma, whereas CGS-1 and -2, as well as BC-1, showed a more restricted distribution. In particular, all 3 antibodies showed strong reactions at the border between the neoplastic cells and stroma.

Castellani *et al.* (1994) have reported that MAb BC-1 recognises the neovasculature in neoplastic as well as in normal tissues. We have obtained identical results with both CGS-1 and -2. Figure 5 shows serial sections of a glioblastoma multiforme with the typical glomerulus-like vascular structures stained by BC-1, CGS-1 and -2. All 3 antibodies gave identical staining patterns. Furthermore, we have previously shown in an exhaustive manner that foetal lung fibroblasts produce more B-FN with respect to foetal skin fibroblasts derived from the same foetuses (Borsi *et al.*, 1992*a*). These data were based on S1 nuclease analysis of RNA as well as on Western blot and immunohistochemical experiments using MAb BC-1. Identical results have now been obtained using CGS-1 and -2 (data not shown).

In immunohistochemical experiments CGS-1 and -2 recognise mouse and chicken B-FN

MAb BC-1 is strictly human-specific. This is a limitation since it does not allow studies on animal models. Since rat and human ED-B have an identical primary structure and there is a 98% homology between human and chicken ED-B (Hynes,

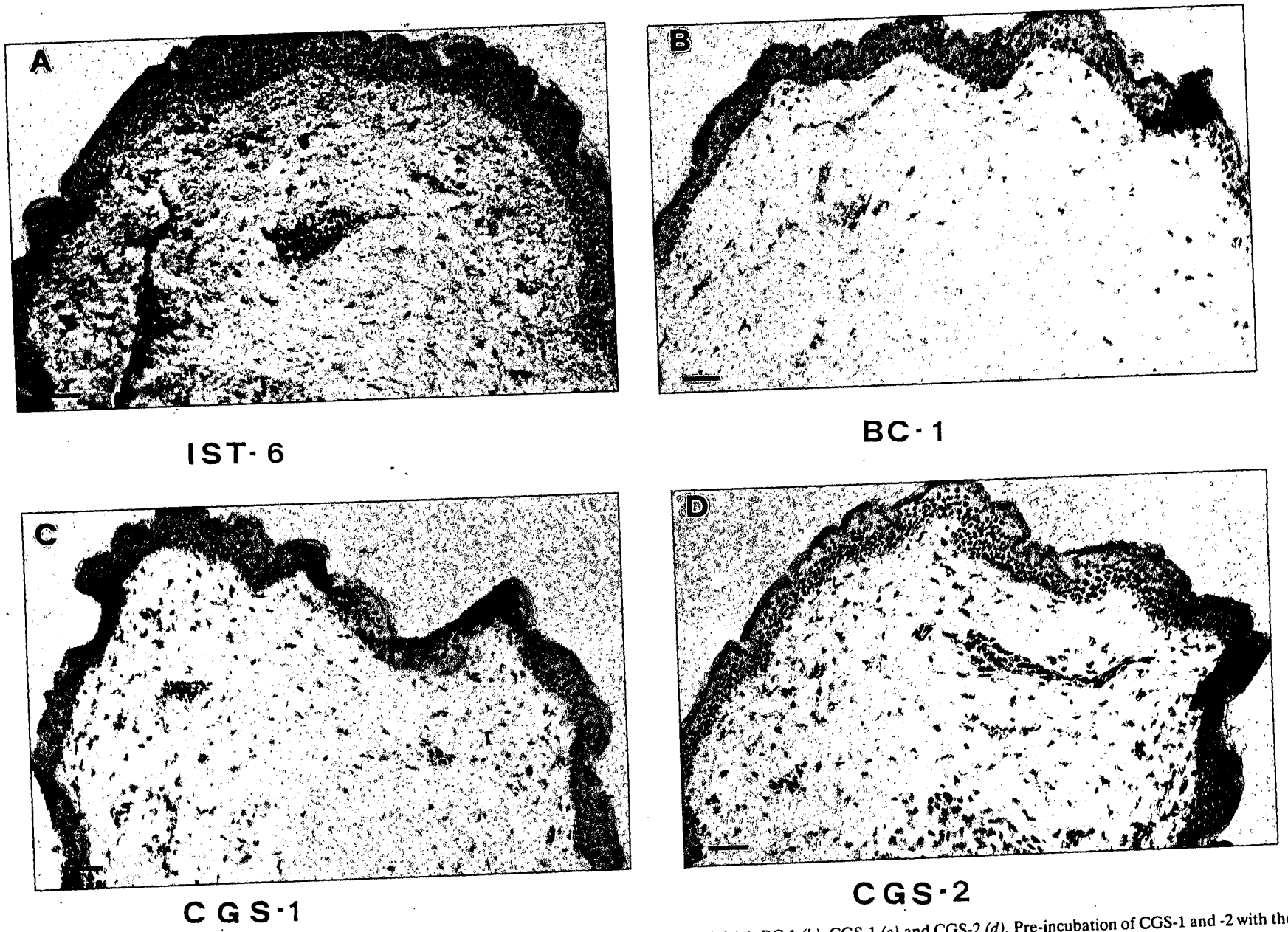

FIGURE 3 – Immunohistochemical experiments on serial sections of normal skin, stained using IST-6 *(a)*, BC-1 *(b)*, CGS-1 *(c)* and CGS-2 *(d)*. Pre-incubation of CGS-1 and -2 with the recombinant ED-B peptide completely inhibited the reaction, while no inhibition was observed using other recombinant FN type III repeats. Scale bar: 10 μm.

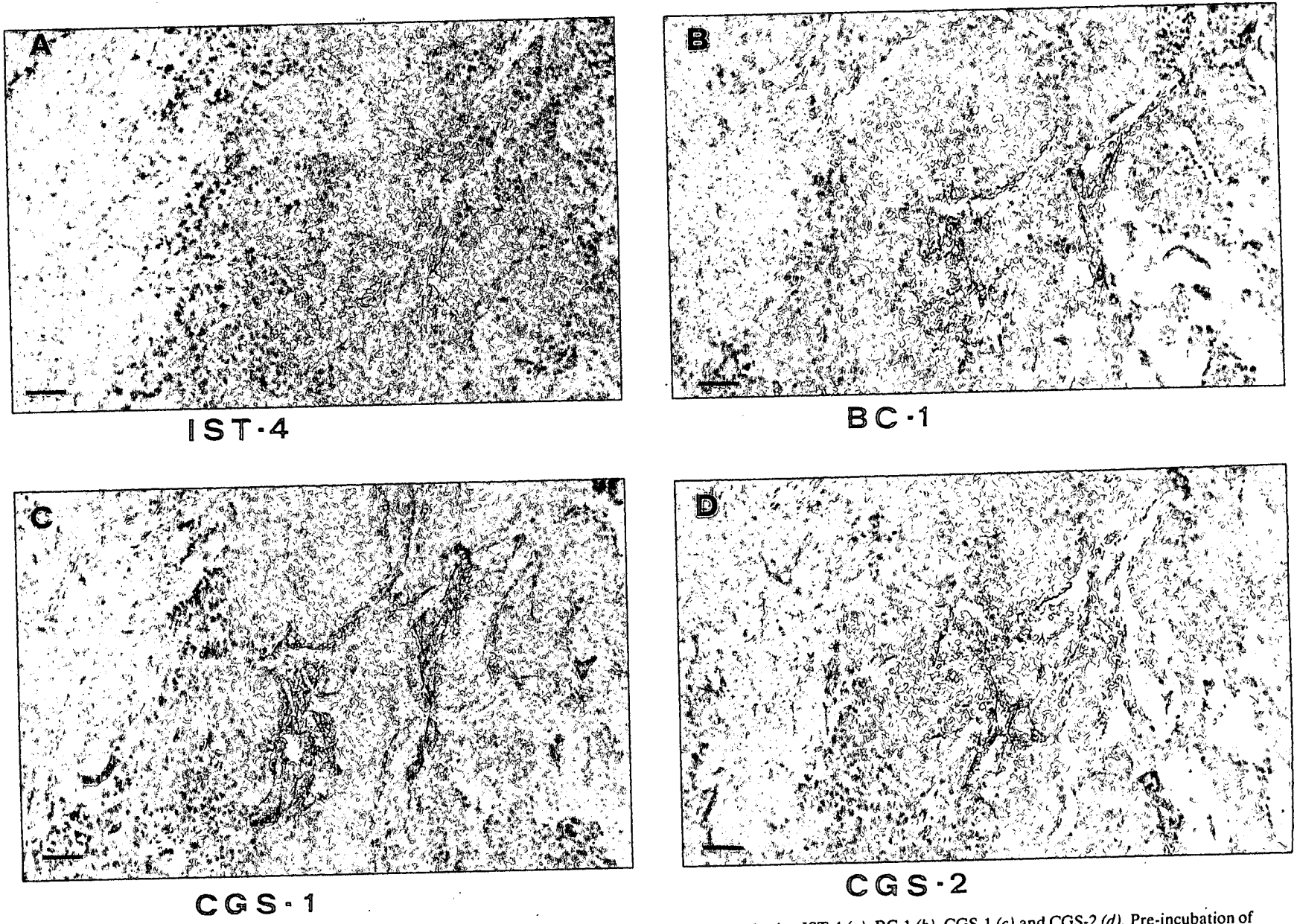

FIGURE 4 – Immunohistochemical experiments on serial sections of an invasive ductal breast carcinoma, stained using IST-4 *(a)*, BC-1 *(b)*, CGS-1 *(c)* and CGS-2 *(d)*. Pre-incubation of CGS-1 and -2 with the recombinant ED-B peptide completely inhibited the reaction, while no inhibition was observed using other recombinant FN type III repeats. Scale bar: 10 μm.

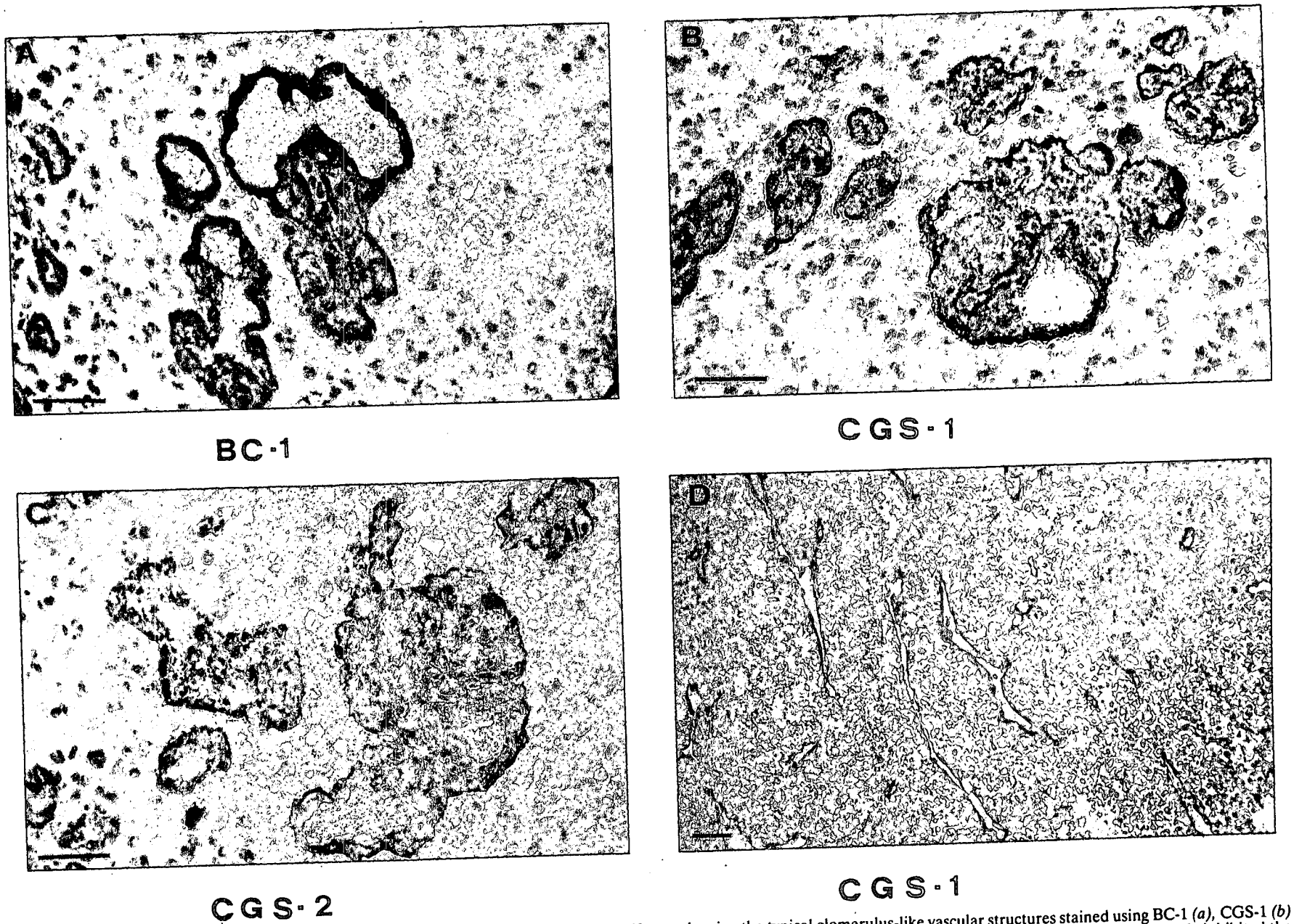

FIGURE 5 – Immunohistochemical experiments on serial sections of glioblastoma multiforme showing the typical glomerulus-like vascular structures stained using BC-1 *(a)*, CGS-1 *(b)* and CGS-2 *(c)* and a section of the mouse teratocarcinoma F9, stained using CGS-1 *(d)*. Pre-incubation of CGS-1 and -2 with the recombinant ED-B peptides completely inhibited the reaction, while no inhibition was observed using other recombinant FN type III repeats. Scale bar: 10 μm.

1990), we expected that CGS-1 and -2 would be able to react with B-FN from these species. In immunohistochemical experiments, CGS-2 reacted with chicken embryos (data not shown) and both CGS-1 and -2 with murine tumours. Figure 5 shows a mouse teratocarcinoma stained using CGS-1. It is possible to recognise a strong reaction with the vascular structures of the neoplasm. In contrast, all of the other normal mouse organs tested (liver, spleen, kidney, stomach, small and large intestine, ovary, uterus, bladder, pancreas, suprarenal glands, skeletal muscle, heart, lung, thyroid and brain) gave no reaction (data not shown).

DISCUSSION

It is difficult to prepare antibodies against highly conserved self-antigens by immunisation. This has prompted the use of phage antibody technology to isolate antibody fragments such as those against the immunoglobulin-binding protein BiP (Nissim *et al.*, 1994) and calmodulin (Griffiths *et al.*, 1994). We now used this technology to isolate 2 human antibody fragments (CGS-1 and CGS-2) against the conserved ED-B oncofoetal domain of FN, a marker of angiogenesis (Castellani *et al.*, 1994).

The CGS-1 and -2 antibody fragments bound directly to the ED-B domain of human B-FN (with affinities of 12 and 2 nM, respectively) and recognised all native and recombinant FN and FN fragments containing the ED-B sequence, without cross-reacting with any of the other type III repeats tested. In immunohistochemical studies on human tissues, the 2 antibodies recognised the same histological structures stained with MAb BC1. However, in contrast with MAb BC-1, these antibody fragments recognised B-FN of other species (as shown for CGS-1 with mouse and CGS-2 with mouse and chicken). Despite the similarities in binding specificity of CGS-1 and -2 for the ED-B domain, these antibody fragments must differ in detailed recognition. Thus, the key loops at the centre of the antigen-binding sites (V_H-CDR3 and V_λ CDR3) differ in sequence: the CGS-1 epitope appears to be located on C-terminal portions of the ED-B domain, whereas the CGS2 epitope is mainly located on the N-terminal portion (Fig. 2). The CGS-2 antibody binds to chicken B-FN, while CGS-1 does not.

These antibody fragments could be valuable for targetting human neovasculature in diagnostic imaging or in therapy, serving as building blocks for engineered human antibodies (of any desired isotype) or for other engineered fragments (Holliger and Winter, 1993). Neovasculature is an attractive target for imaging and therapy as the antigens are accessible and should be bound rapidly and extracellular matrix antigens can be very stable, allowing a long antibody residence time on tumours (Riva *et al.*, 1994). The possibility of using a range of engineered antibodies should facilitate the development of antibody reagents with suitable pharmacokinetics, valency, functional affinity and effector functions. As the CGS-1 and -2 antibody fragments also bind to B-FN of other species, the availability of animal models should further facilitate the evaluation of engineered antibodies (or fragments) for neovasculature targetting in humans.

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REFERENCES

BETTER, M., CHANG, C.P., ROBINSON, R.R. and HORWITZ, A.H., *Escherichia coli* secretion of an active chimeric antibody fragment. *Science*, **240**, 1041–1043 (1988).

BIRD, R.E., HARDMAN, K.D., JACOBSON, J.W., JOHNSON, S., KAUFMAN, B.M., LEE, S.M., LEE, T., POPE, S.H., RIORDAN, G.S. and WHITLOW, M., Single-chain antigen-binding proteins. *Science*, **242**, 423–426 (1988).

BORSI, L., BALZA, E., ALLEMANNI, G. and ZARDI, L., Differential expression of the fibronectin isoform containing the ED-B oncofetal domain in normal human fibroblast cell lines originating from different tissues. *Exp. Cell Res.*, **199**, 98–105 (1992*a*).

BORSI, L., CARNEMOLLA, B., CASTELLANI, P., ROSELLINI, C., VECCHIO, D., ALLEMANNI, G., CHANG, S.E., TAYLOR-PAPADIMITRIOU, J., PANDE, H. and ZARDI, L., Monoclonal antibodies in the analysis of fibronectin isoforms generated by alternative splicing of mRNA precursors in normal and transformed human cells. *J. Cell Biol.*, **104**, 595–600 (1987).

BORSI, L., CARNEMOLLA, B., NICOLÒ, G., SPINA, B., TANARA, G. and ZARDI, L., Expression of different tenascin isoforms in normal, hyperplastic and neoplastic human breast tissues. *Int. J. Cancer*, **52**, 688–692 (1992*b*).

CARNEMOLLA, B., BALZA, E., SIRI, A., ZARDI, L., NICOTRA, M.R., BIGOTTI, A. and NATALI, P.G., A tumor-associated fibronectin isoform generated by alternative splicing of messenger RNA precursors. *J. Cell Biol.*, **108**, 1139–1148 (1989).

CARNEMOLLA, B., LEPRINI, A., ALLEMANNI, G., SAGINATI, M. and ZARDI, L., The inclusion of the type III repeat ED-B in the fibronectin molecule generates conformational modifications that unmask a cryptic sequence. *J. biol. Chem.*, **267**, 24689–24692 (1992).

CASTELLANI, P., SIRI, A., ROSELLINI, C., INFUSINI, E., BORSI, L. and ZARDI, L., Transformed human cells release different fibronectin variants than to normal cells. *J. Cell Biol.*, **103**, 1671–1677 (1986).

CASTELLANI, P., VIALE, G., DORCARATTO, A., NICOLÒ, G., KACZMAREK, J., QUERZÉ, G. and ZARDI, L., The fibronectin isoform containing the ED-B oncofetal domain: a marker of angiogenesis. *Int. J. Cancer*, **59**, 612–618 (1994).

FFRENCH-CONSTANT, C., VAN DE WATER, L., DVORAK, H.F. and HYNES, R.O., Reappearance of an embryonic pattern of fibronectin splicing during wound healing in the adult rat. *J. Cell Biol.*, **109**, 903–914 (1989).

FOLKMAN, J., Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nature (Med.)*, **1**, 27–31 (1995).

GLOCKSHUBER, R., STADLMÜLLER, J. and PLUCKTHUN, A., Mapping and modification of an antibody hapten binding site: a site-directed mutagenesis study of McPC603. *Biochemistry*, **30**, 3049–3054 (1991).

GRIFFITHS, A.D. and 17 OTHERS, Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J.*, **13**, 3245–3260 (1994).

HOLLIGER, P. and WINTER, G., Engineering bispecific antibodies. *Curr. Opin. Biotechnol.*, **4**, 446–449 (1993).

HUSTON, J.S., LEVINSON, D., MUDGETT, H.M., TAI, M.S., NOVOTNY, J., MARGOLIES, M.N., RIDGE, R.J., BRUCCOLERI, R.E., HABER, E., CREA, R. and OPPERMANN, H., Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli*. *Proc. nat. Acad. Sci. (Wash.)*, **85**, 5879–5883 (1988).

HYNES, R.O., *Fibronectins*, Springer-Verlag, New York (1990).

KACZMAREK, J., CASTELLANI, P., NICOLÒ, G., SPINA, B., ALEMANNI, G. and ZARDI, L., Distribution of oncofetal fibronectin isoforms in normal, hyperplastic and neoplastic human breast tissues. *Int. J. Cancer*, **58**, 11–16 (1994).

KORNBLIHTT, A.R., UMEZAWA, K., VIBE-PEDERSEN, K. and BARALLE, E., Primary structure of human fibronectin: differential splicing may generate at least 10 polypeptides from a single gene. *EMBO J.*, **4**, 1755–1759 (1985).

LEPRINI, A., QUERZÉ, G. and ZARDI, L., Tenascin isoforms: possible targets for diagnosis and therapy of cancer and mechanisms regulating their expression. *Perspect. Develop. Neurobiol.*, **2**, 117–123 (1994).

MCCAFFERTY, J., GRIFFITHS, A.D., WINTER, G. and CHISWELL, D.J., Phage antibodies: filamentous phage displaying antibody variable domains. *Nature (Lond.)*, **348**, 552–554 (1990).

NERI, D., PETRUL, H., LIGHT, Y., MARAIS, R., BRITTON, K.E., WINTER, G. and CREIGHTON, A.M., Radioactive labeling of recombinant antibody fragments by phosphorylation using human casein kinase II and [g-32P]-ATP. *Nature (Biotechnology)* (1996) In press.

NISSIM, A., HOOGENBOOM, H.R., TOMLINSON, I.M., FLYNN, G., MIDGLEY, C., LANE, D. and WINTER, G., Antibody reagents from a "single-pot" phage display library as immunochemical reagents. *EMBO J.*, **13**, 692–698 (1994).

NORTON, P.A. and HYNES, R.O., Alternative splicing of chicken fibronectin in embryos and in normal and transformed cells. *Mol. cell. Biol.*, **7**, 4297–4307 (1987).

OYAMA, F., HIROHASHI, S., SHIMOSATO, Y., TITANI, K. and SEKIGUCHI, K., Deregulation of alternative splicing of fibronectin pre-mRNA in malignant human liver tumors. *J. biol. Chem.*, **264**, 10331–10334 (1989).

PETERS, J.H., TREVITHICK, J.E., JOHNSON, P. and HYNES, R.O., Expression of the alternatively spliced EIIIB segment of fibronectin. *Cell Adhesion Comm.*, **3**, 67–89 (1995).

RISAU, W. and LEMMON, V., Changes in the vascular extracellular matrix during embryonic vasculogenesis and angiogenesis. *Develop. Biol.*, **125**, 441–450 (1988).

RIVA, P., ARISTA, A., TISON, V., STURIALE, C., FRANCESCHI, G., SPINELLI, A., RIVA, N., CASI, M., MOSCATELLI, G. and FRATTARELLI, M., Intralesional radio-immunotherapy of malignant gliomas. *Cancer*, **73**, 1076–1082 (1994).

SKERRA, A. and PLUCKTHUN, A., Assembly of a functional immunoglobulin Fv fragment in *Escherichia coli*. *Science*, **240**, 1038–1041 (1988).

SMITH, G.P., Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science*, **228**, 1315–1317 (1985).

VAN DEN HOFF, A., Stromal involvement in malignant growth. *Adv. Cancer Res.*, **50**, 159–196 (1988).

VARTIO, T., LAITINEN, L., NARVANEN, O., CUTOLO, M., THORNELL, L.E., ZARDI, L. and VIRTANEN, I., Differential expression of the ED sequence-containing form of cellular fibronectin in embryonic and adult human tissues. *J. Cell Sci.*, **88**, 419–430 (1987).

WILLIAMS, S.C. and WINTER, G., Cloning and sequencing of human immunoglobulin V_λ gene segments. *Europ. J. Immunol.*, **23**, 1456–1461 (1993).

WINTER, G., GRIFFITHS, A.D., HAWKINS, R.E. and HOOGENBOOM, H.R., Making antibodies by phage display technology. *Ann. Rev. Immunol.*, **12**, 433–455 (1994).

ZARDI, L., CARNEMOLLA, B., SIRI, A., PETERSEN, T.E., PAOLELLA, G., SEBASTIO, G. and BARALLE, F.E., Transformed human cells produce a new fibronectin isoform by preferential alternative splicing of a previously unobserved exon. *EMBO J.*, **6**, 2337–2342 (1987).